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The polarized architecture of hepatocytes

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The polarized architecture of hepatocytes

Regulation by intrinsic and extrinsic factors

Christiaan Lucas Slim

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CHAPTER 1

Introduction and scope of the thesis

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INTRODUCTION

This thesis focuses primarily on the development and maintenance of the polarized architecture of hepatocytes, the main epithelial cell type in the liver. Before going into hepatocyte polarity and the liver tissue architecture, it is important to first introduce the topic of epithelial cell polarity and its design principles. The following paragraphs describe the basic concepts of epithelial cells, their polarized architecture, and some of the design principles that epithelial cells use to form epithelial tissues.

The polarized architecture of epithelial cells

Epithelial cells constitute the biggest cell pool in the mammalian organism, with ~60% of mammalian cell types being of epithelial or epithelial-derived origin [1]. Epithelial cells are situated at the interface between the organisms exterior and interior environment and are found in a wide variety of epithelial tissues, such as in the skin, lung, liver, gut, and kidney. The major function of the epithelium is to protect the organism's interior milieu by physically separating it from the exterior environment, and also to regulate the transport of molecules (e.g., nutrients) between the environments [1,2]. For example, the intestinal epithelium regulates the selective uptake of nutrients from digested food, while at the same time protecting the organism's interior from the plethora of bacterial species that inhabit the intestinal lumen. In the lungs, the epithelium allows for the exchange of gaseous molecules over the epithelium, while contemporaneously keeping hazardous materials (e.g., dust and bacteria) out of blood. In the liver, hepatocytes effectively separate blood and bile compartments. Epithelium can be classified into various types by the shape of individual cells and the tissue anatomy (Figure 1). In squamous epithelial tissues the cells are wider then they are tall (i.e., they appear 'flat'), while in columnar epithelium the cells are taller than they are wide. Cuboidal epithelial cells have roughly the same height and width. These three types of epithelial cells can be found in two different tissue architectures: stratified and non-stratified. In stratified epithelium, cells are arranged in multiple layers, while in non-stratified epithelium cells are aligned in single-cell thick (mono)layers. Notably, non-stratified (monolayered) epithelium is the most abundant type of epithelium in mammalian organisms and is commonly referred to as 'simple' epithelium.

Simple epithelial cells are polarized cells in the sense that they create specific plasma membrane domains (also referred to as surfaces) that face either the exterior or the interior environment, or neighboring cells (Figure 2A). The apical membrane faces

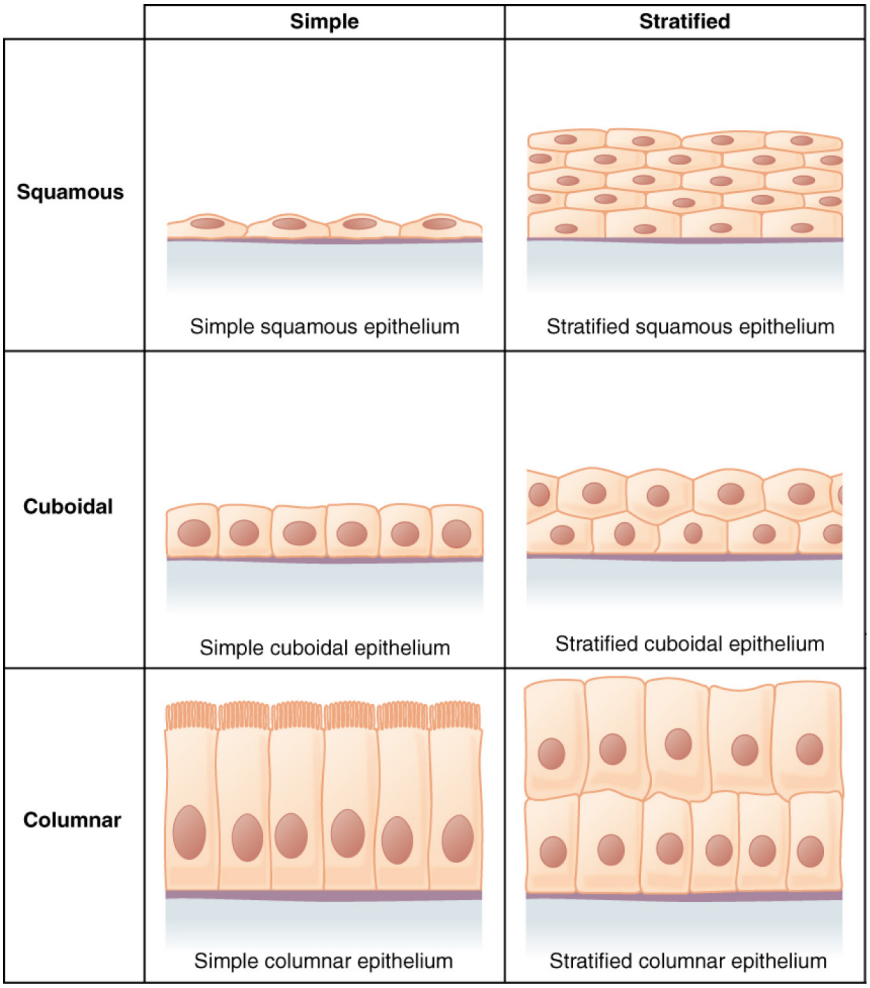

Figure 1. Epithelial cells and their various forms. Simple (i.e., non-stratified) epithelial cells form a single-cell sheet or monolayer, while stratified epithelium is composed of multiple layers of cells. Reused with permission from OpenStax College (2013) [130].

the external environment or 'lumen' of the organism, such as the interior of the gut or lung. The basal membrane faces the interior milieu of the organism and is typically in contact with the extracellular matrix (ECM; explained in more detail below) and, ultimately, underlying blood vessels [1]. The lateral plasma membrane domains contact neighboring cells and contains cell-cell adhesion protein complexes like tight junctions, adherens junctions and desmosomes [3]. The apical and basal-lateral (better known as basolateral) plasma membrane domains are separated, and their specific lipid and protein compositions maintained, by tight junctions [3–5], which are also important for

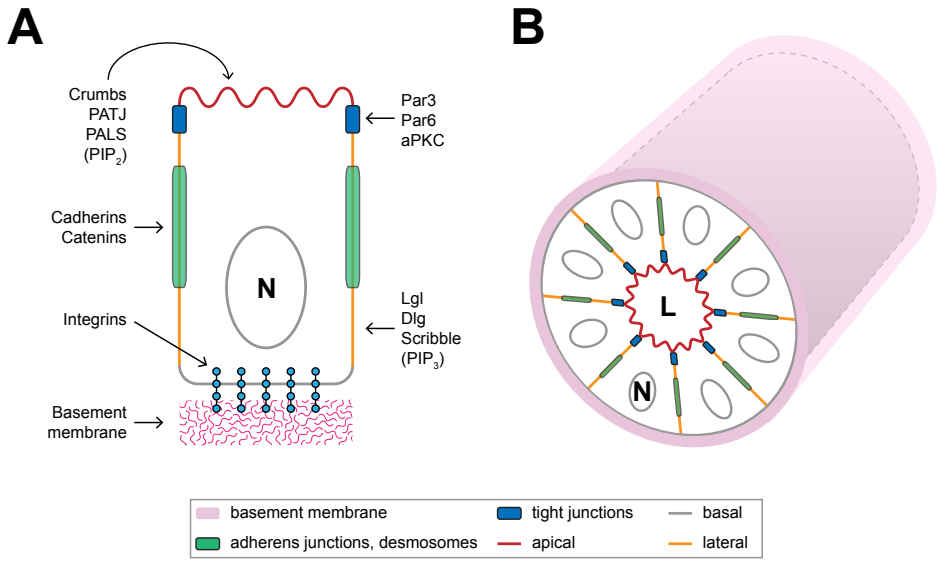

Figure 2. Simple epithelial cell polarity and tubular architecture. **(A)** Simple epithelial cells are polarized. Their apical, lateral, and basal plasma membrane domains are composed of specific sets of proteins and lipids. Apical and basal-lateral plasma membrane domains are separated by tight junctions to prevent leaking of plasma membrane components and limits paracellular transport. Basal plasma membrane domains are in contact with a basement membrane or basal lamina and provide cell attachment. Adherens junctions and desmosomes generate cell-cell adhesion between neighboring epithelial cells. **(B)** Simple epithelial cells are aligned in monolayers that surround a central lumen, and form tubular structures. L = lumen. N = nucleus.

barrier functions of the epithelium by limiting paracellular transport. Notably, apical-basal polarity allows for vectorial and controlled transport between the interior and exterior environments.

Research in the field of epithelial polarity has largely focused on how cells generate apical-basal polarity and how they subsequently maintain their polarity and arrange themselves into an epithelial organ. It has become clear that the integration of 'polarity cues' from various biological sources are key to forming polarized epithelial tissue. For example, the spatially and temporally coordinated interactions between cells (via cadherins and tight junctions) and the ECM (via integrins) are crucial for the formation of apical-basal polarity [1,2,6]. Downstream of these 'outside-in' signaling molecules is a vast network of regulatory proteins such as GTPases and their effectors which translate polarity cues into 'polarizing' behavior that includes the generation of the above described specialized plasma membrane domains (apical, lateral, basal) by polarized (i.e., targeted) protein and lipid trafficking [7–12] and the asymmetrical distribution of protein complexes [1,13,14]. Important protein complexes involved in generating apical-

basal polarity typically include the apically localized Crumbs-Patj (PALS1-associated tight junction protein) - PALS (proteins associated with Lin-7) complex, the tight-junctional Par3 (partioning defective 3) - Par6 - aPKC (atypical protein kinase C) complex, and the lateral Lgl (lethal giant larvae) - Dlg (discs large)- Scribble complex [15–18]. Most members of these protein complexes are in reciprocal interaction with each other, mutually excluding members of other complexes from their plasma membrane domain by phosphorylation [14,19]. Apart from these protein complexes, membrane identity is also conferred by lipid composition such as the polarized localization of phosphorylated phosphatidylinositols (PtdIns). PtdIns(4,5)P_2 (or PIP2) is an apical determinant, while PtdIns(3,4,5)P_3 (or PIP3) is generally found at the basolateral plasma membrane domain in epithelial cells (Figure 2A) [12,20]. Generating the polarized localization of protein and lipid complexes relies heavily on polarized protein and lipid trafficking. Polarized trafficking is therefore both cause and consequence of epithelial (apical-basal) polarity, as it is needed for the initial asymmetric distribution of proteins and lipids in the cell and the initial formation of the distinct plasma membrane domains during apical-basal polarity development, but also reinforces and maintains the polarized state [11,21]. Thus, epithelial tissues are important for protection and communication between the internal and external environment of the organism, and are comprised of epithelial cells that exhibit apical-basal polarity. Apical-basal polarity is developed and maintained by selective protein and lipid homeostasis at the plasma membrane.

Simple epithelial cells form tubes with a single central lumen

By using the apical-basal polarity axis, individual simple epithelial cells position themselves into sheets or monolayers that surround a central luminal cavity, with all apical plasma membrane domains facing inwards into the lumen, lateral surfaces contacting neighboring cells, and basal surfaces facing the extracellular matrix and blood (Figure 2B). By doing so, they efficiently develop a protective and regulatory barrier between the inside and outside environment of the organism. Ultrastructurally, these monolayers develop into tubes with a single central lumen (e.g., as found in the lung and kidney) [1]. Simple epithelial cells form lumens via a variety of mechanisms such as cavitation or hollowing [1,22], though a single central lumen is eventually always created. During initial development of the lumen, simple epithelial cells are committed to the formation of only one apical domain, which becomes the main target for apical protein trafficking and secretion, mediated by polarity and trafficking proteins such as Rab11a, Rab8a, Cdc42, and synaptotagmin-like protein 2a (Slp2a) [22–24]. Notably, inhibiting the function of Slp2a in Madin-Darby canine kidney (MDCK) cells (a model

for simple epithelium) induces the formation of multiple apical domains per cell and results in malformed simple epithelial structures without a single central lumen [24]. Thus, simple epithelial cells use their apical-basal polarity axis as an internal guide for polarized protein trafficking to allow the formation of tubular structures with a single central lumen.

Shaping tissues with orientated cell divisions

Because epithelial cells use their apical-basal polarized state as an internal compass to coordinate the generation of epithelial monolayers and tubes, and the vectorial transport of molecules between the different body compartments, it is important that apical-basal polarity is not compromised during development and maintenance of these tissues. Apart from protein trafficking, epithelial cells utilize other mechanisms for maintaining apical-basal polarity within the tissue architecture. One of these mechanisms connects to a fundamental aspect of the developing organism: cell division. During the growth of an epithelial organ, apical-basal polarized epithelial cells divide to increase the width or length of the tissue or tube. Cell divisions, however, are not random in these tissues, but tightly controlled and are known as oriented cell divisions. The orientation of cell division is a well-known concept in the field of developmental biology and has been subject of numerous excellent reviews [25–32]. The orientation of cell division is commonly known for its importance in the generation of multiple cell types during development of the organism via a process known as 'asymmetric' cell division (see paragraph below, Figure 3), though orientation of cell division is also used by tube-forming epithelial cells to develop and maintain apical-basal polarity and tubular integrity [14,22,23,28]. As described below in more detail, the orientation of cell division relies on the orientation of the mitotic spindle during mitosis, which determines the position of the cleavage furrow and, thus, how cells divide their plasma membrane domains and how individual daughter cells are positioned within the epithelial tissue. Initially, it was believed that cell shape alone was responsible for dictating the orientation of the mitotic spindle and cell division, as according to the rule of Sachs and Hertwig, the spindle axis normally orients itself along the longest axis of the cell [27,33,34]. This view does not hold true anymore, however, as many studies have repeatedly indicated that the orientation of cell division is in many cases regulated on the molecular level by specific localization and activity of conserved protein complexes at the plasma membrane. Thus, cell divisions are oriented and controlled, and function as an important mechanism for developing and maintaining tissue architecture.

Protein complexes in asymmetric cell division

One of the earliest studied proteins involved in mitotic spindle orientation and cell division are the partitioning-defective (PAR) proteins, originally discovered in worms, and found to regulate a so-called 'anterior-posterior' cortical polarity axis (Figure 3) [35]. The first division in worms produces daughter cells that are unequal in size because of the polarized distribution of PAR protein complexes (Par3-Par6-PKC3) that results in unequal positioning of mitotic spindle orienting proteins such as Leu-Gly-Asn–enriched protein (LGN, or GPR-1/2 in worm, and sometimes also referred to as G-protein signaling modulator 2), and nuclear mitotic apparatus protein (NuMA, or LIN-5 in worm). The polarized distribution of these proteins generates unequal force on the mitotic spindle and results in unequally sized daughter cells after cytokinesis (Figure 3, worm embryo) [14,35–37]. LGN and NuMA are part of an evolutionary conserved G-protein signaling pathway across metazoan, including mammalian epithelial cells [26,38,39].

In flies, neuroblasts are crucial for the formation of the fly nervous system, and are widely used as a model system for neurogenesis and asymmetric cell division. Neuroblasts divide asymmetrically to produce daughter cells that, positioned away from the epithelial neuroblast niche, differentiate into ganglion mother cells and ultimately neurons and glial cells. Disruption of asymmetric cell division results in impaired neurogenesis and brain abnormalities [40]. In this model, the PAR polarity complex Par3-Par6-aPKC and spindle orienting complex Inscutable-LGN-NuMA (Insc-Pins-Mud in flies) are localized apically and induces a mitotic spindle pole orientation towards the Insc-LGN-NuMA 'patch', resulting in a mitotic spindle that is aligned with the apical-basal axis of the neuroblast. Subsequent cytokinesis results in cleavage furrow formation perpendicular to the apical-basal polarity axis (that is, aligned with the substratum), asymmetric cell division, and positioning of one of the daughter cells away from the neuroblast (Figure 3, fly neuroblast). The apical proteins complexes are retained by the apically positioned neuroblast cell in which aPKC promotes self-renewal. Proteins localized to the basal plasma membrane domain of the neuroblast during cell division (e.g., Miranda, Prospero, and Brain tumor) regulate differentiation of the subsequently basally positioned daughter cell into neural cells [29]. Another example of asymmetric cell division is found in the mouse skin, where basal skin cells early in development divide symmetrically to increase basal cell population, while later divide asymmetrically to produce differentiated skin cells and the skin layer [41]. Again, the apically localized LGN-NuMA protein complex is indispensable for coupling the orientation of the mitotic spindle with the apical-basal polarity axis of the cell [41–44], and thereby driving the orientation of cell division and tissue architecture (Figure 3, mouse epidermis). Thus,

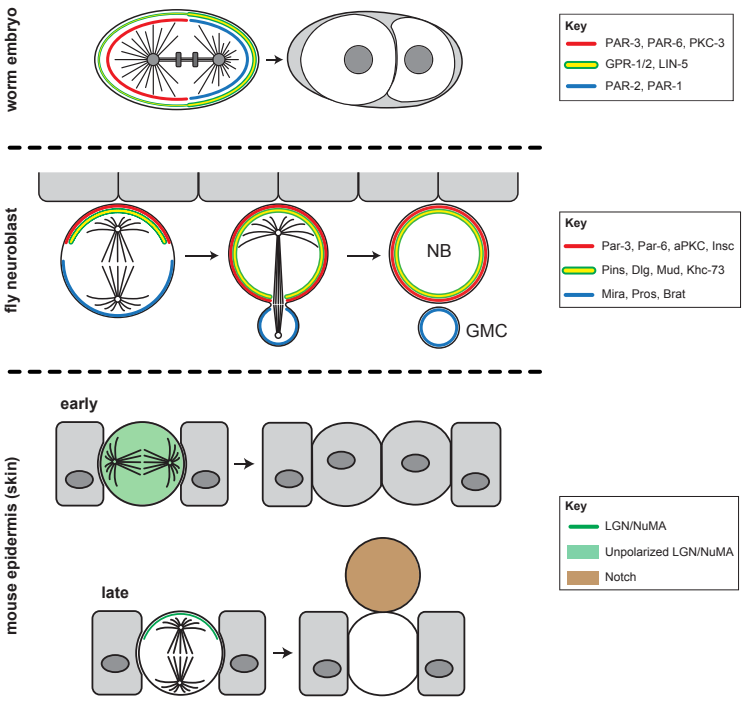

Figure 3. Asymmetric cell division in model organisms. In the worm embryo, the first cell division yield daughter cells of unequal size. Par-3, Par-6, and PKC-3 localize to the anterior domain of the cell, while Par-2, Par-1 are positioned to the posterior end. Mutual phosphorylation generates and maintains asymmetrical protein localization. GPR-1/2 (LGN) and LIN-5 (NuMA) favor the posterior end, and are responsible for the force generation on astral microtubules resulting in movement of the mitotic spindle towards the posterior end of the cell. Subsequent cleavage furrow formation and cytokinesis results in unequally sized daughter cells. In the fly neuroblast (NB), NB divide asymmetrically to produce a new NB and a ganglion mother cell (GMC). Apical Pins (LGN), Dlg, Mud (NuMA), and the kinesin Khc-73 orient the mitotic spindle to asymmetrically segregate apical and basal determinants. Par-3, Par-6, aPKC, and Inscutable control NB cell fate, while Miranda (Mira), Prospero (Pros), and Brain tumor (Brat) control GMC cell fate. In the mouse epidermis, early cell divisions of basal cells are symmetric and function to increase the basal cell monolayer mass. Late cell divisions are asymmetrical, mediated by apically localized LGN/NuMA mitotic spindle orientation, to produce the skin layer (stratification). Reproduced with permission of Company of Biologists from [29], © 2013.

protein complexes that regulate apical-basal polarity, such as the PAR protein complex, and spindle-orienting proteins such as LGN and NuMA, cooperatively control the (a) symmetry of cell division, and indicates that apical-basal polarity is intimately linked with orientation of cell division.

Orienting the mitotic spindle

How does the polarized distribution of cortical proteins such as LGN and NuMA translate into orientation of the mitotic spindle? Two spindle orienting pathways, mainly developed in fly neuroepithelium (see previous paragraph), currently dominate the

field [29,45]. Both pathways converge on the in mitosis cortically localized protein LGN. The first pathway involves a protein complex comprised of LGN, Dlg, and the kinesin motor protein Kinesin-73. In this model, Dlg localizes with the apically polarized localization of LGN, and acts as an adaptor molecule that links cortical LGN with Kinesin-73 and thereby orienting the mitotic spindle via a 'spindle capture' mechanism [46]. Loss of any of these components results in misorientation of the mitotic spindle. The second pathway contains a complex between LGN, NuMA, and dynein [26,32,47–50]. NuMA is recruited by LGN (by interaction with the N-terminal tetratricopeptide repeat (TPR) domain on LGN) to the cell cortex, which recruits dynein and generates force on the mitotic spindle's astral microtubules to orient the mitotic spindle [48]. NuMA binding to LGN is regulated by the heterotrimeric G-protein subunit Gα (mainly the i-subunit). Binding of Gα to the GoLoco motifs on LGN removes an auto-inhibitory conformational loop between the TPR domain and GoLoco domains, allowing NuMA to interact with LGN [51–54]. NuMA also localizes to spindle poles and microtubules, where it is involved in microtubule maintenance [47,55]. Thus, similarly to Dlg, NuMA acts as an adaptor protein that links LGN with a molecular motor, dynein, to generate force on astral microtubules and orient the mitotic spindle. Importantly, the position of LGN is crucial for determining the orientation of the mitotic spindle and the outcome of cell division.

Simple epithelial cells symmetrically divide plasma membrane domains

Following the work in flies and worm, it was recently discovered that orientation of cell division is necessary for the development and maintenance of mammalian epithelial monolayers and tubes as well, and failure to properly orient the mitotic spindle in epithelial tissues correlates with tumor genesis [1,56–60]. Interestingly, in contrast to the asymmetric cell divisions described above, epithelial cells predominantly divide symmetrically and keep their individual plasma membrane domain identities intact [61]. That is to say, epithelial cells symmetrically segregate their apical and basolateral membrane domains, and proteins and lipids, to both daughter cells. Subsequently, both of the daughter cells are positioned in the plane of the monolayer (Figure 4). Interestingly, the protein complexes that function in asymmetric cell division and spindle orientation are evolutionary conserved and also function to orient the mitotic spindle in symmetrically dividing epithelial cells. In 2001, the Macara lab identified the mammalian homologue of LGN [62]. In MDCK monolayers, LGN was found to colocalize with NuMA at the lateral cell cortex as was predicted from other model systems, and overexpression or knockdown of LGN resulted in disorganization of the mitotic spindle [62]. Later, they reported that, as expected from earlier work in model systems, Gα

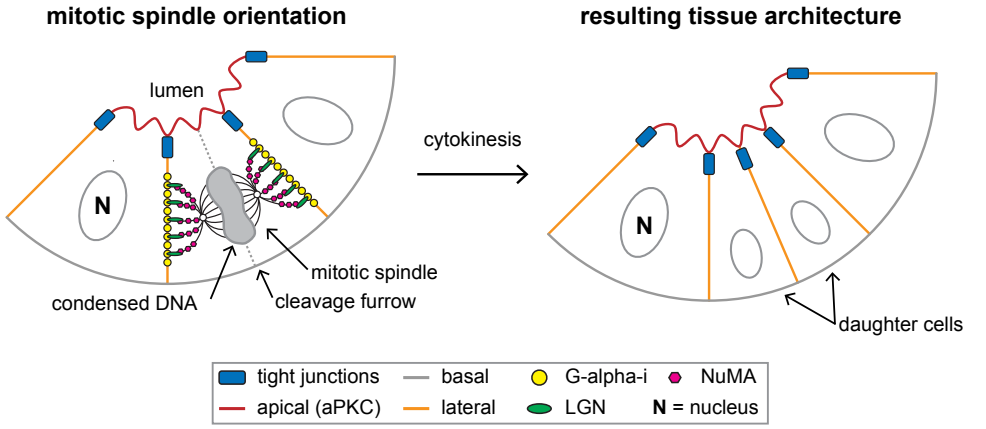

Figure 4. Simple epithelial cells divide symmetrically to develop and maintain tissue architecture. During mitosis, G-alpha-i acquires a selective lateral localization and recruits LGN to the cortex. Apical aPKC (not shown) phosphorylation of LGN inhibits LGN recruitment to the apical surface. LGN interacts with NuMA-positive astral microtubules to orient the mitotic spindle via the microtubule motor dynein (not shown). Upon cytokinesis, the cleavage furrow aligns with the apical-basal axis and symmetrically segregates plasma membrane domains to both daughter cells.

recruits LGN to the lateral cortex. In addition to MDCK cells, Morin and colleagues reported that the Gα/LGN/NuMA protein pathway also functions in maintaining the progenitor pool of neuroepithelial cells in chickens [63,64]. The role for Gα in recruiting the LGN/NuMA/dynein protein complex was made more convincing by Woodard and colleagues who reported that knockdown of Ric-8a, a guanine exchange factor (GEF) for Gαi, perturbs the cortical recruitment of LGN/NuMA/dynein in HeLa cells and MDCK monolayers, resulting in misorientation of the mitotic spindle [65].

MDCK cells can be cultured on a basement-membrane extract (for example Matrigel) which mimics the basal lamina on which epithelial cells normally sit upon (explained in more detail below). In these models, cells form cysts or spheroids comprised of a monolayer of cells surrounding a central lumen, thereby mimicking the terminal branches of tubular epithelial organs [2]. Zheng and colleagues elegantly showed that knockdown of LGN in MDCK cysts resulted in the formation of cysts with multiple small lumens, evidently caused by mitotic spindle misorientation [66]. In addition, inhibiting the interaction of LGN with either NuMA or Gαi also resulted in spindle misorientation and abnormal cystogenesis. During mitosis, LGN localizes to the lateral membrane. Using a Crumbs3 apical targeting vector, they efficiently forced Gαi to the apical surface during mitosis, which, indeed, resulted in the recruitment of LGN to the apical surface and misalignment of the mitotic spindle, perturbing symmetric cell division and inheri-

tance of plasma membrane domains, and normal cyst formation [66]. In another study, silencing of the PAR protein Par3 resulted in spindle misorientation and perturbed normal cyst formation in MDCK cells [67]. Knockdown of Par3 also disrupted the apical localization of aPKC, while forced localization of aPKC to the apical surface was able to rescue the Par3 silencing phenotype. Interestingly, LGN was also mislocalized to the apical surface under Par3 knockdown conditions, implying a role for Par3 and aPKC in excluding LGN from the apical surface during mitosis. Indeed, aPKC was shown to increase phosphorylation of LGN, which recruited the polarity protein 14-3-3 (Par5) to LGN, effectively inhibiting the binding of Gαi to LGN (which recruits LGN to the cortex), resulting in exclusion of LGN from the apical surface [67].

In another series of experiments, the small Rho GTPase Cdc42, important for the generation of apico-basal polarity [20] and localized apically, was shown the be important for proper mitotic spindle orientation in human intestinal Caco-2 cysts and frog neural tube cells [68,69], and also in non-polarized adherent HeLa cells [70]. Additionally, silencing of Cdc42 GEFs Tuba and Intersectin 2 was sufficient to misorient the mitotic spindle and inhibit cyst formation in MDCK cells [71,72]. Later, downstream proteins in the Cdc42-mediated spindle orientation pathway were identified as Par6b and aPKC [73]. In this model, Par6b is an apical protein that recruits aPKC to the apical surface, while aPKC protects Par6b from degradation. Par6b was able to rescue Cdc42-knockdown mediated spindle misorientation, while a Cdc42-binding deficient Par6b could not, implying Par6b functions downstream of Cdc42 [73]. Thus, apically Cdc42 regulates the apical recruitment of Par6b, which in turn recruits aPKC to the apical membrane. As described above, aPKC mediates exclusion of LGN from the apical domain so that the mitotic spindle aligns with the plane of the monolayer (i.e., perpendicular to the apico-basal axis), resulting in symmetrical inheritance of plasma membrane domains and proper cyst/tube formation.

Taken together, these studies suggest a model where the mitotic spindle-orienting protein complex LGN/NuMA/dynein is crucial for mitotic spindle alignment perpendicular to the apical-basal polarity axis, and symmetric segregation of plasma membrane domains in simple epithelial cells (Figure 4). Proper localization of this complex to the lateral cortex seems to rely in part on the activity of aPKC in phosphorylating LGN to exclude it from the apical cortex. Confusingly, aPKC and LGN are both apical proteins in asymmetrically dividing cells, complicating the role of aPKC in regulating LGN localization. Other proteins such as Inscutable may function in regulating the activity of aPKC and localization of LGN in both systems [29,31]. Overall, these studies indicate that

simple epithelial cells use their apical-basal polarity axis as an internal guide to control cell division orientation mechanisms and, ultimately, simple epithelial tube formation.

The microenvironment controls simple epithelial architecture

Simple epithelial cells reside on an ECM called the basal lamina (also known as the basement membrane, Figure 2) [1,2,19,74]. The basal lamina mainly consists of laminin and collagen type IV which organize into stiff sheets by crosslinking activity of nidogen (also known as entactin) and heparan sulfate proteoglycan, and gives mechanical support to the epithelial cells. Basement membrane extracts such as Matrigel are now often used to culture epithelial cell lines, in which cells form three dimensional structures that resemble cysts or tubules found in vivo [2]. From these experiments it has become clear that the basement membrane, especially laminin, is important for development of the apical-basal polarity axis and epithelial morphogenesis [6,75–78]. For example, when MDCK cells are cultured on basement membrane substrates, they selectively position their apical domain at the free surface opposite to the substrate-contacting basal domain via signaling through integrins and GTPases such as Rac1 and RhoA [2,6,79,80]. MDCK cells also deposit their own ECM proteins, mostly laminin, which become 'trapped' only at the substrate-containing site and assemble into a basal lamina type ECM [81–83]. The basement membrane is also an important regulator of the orientation of cell division. Integrins, ECM-binding proteins involved in outside-in polarity signaling (cf. Figure 2A), are required for normal spindle alignment in non-polarized cells [84] and in polarized skin cells [41]. In the mouse skin, β1-integrin signaling regulates the proper localization of aPKC, Par3, LGN, and Inscutable, and the NuMA-dynein/dynactin complex [41]. Even in non-polarized HeLa cells the ECM controls mitotic spindle orientation, although it does so by distinct mechanisms and controls mitotic spindle orientation by regulating the cortical distribution of actin binding proteins and crosslinkers such as ezrin [85]. Thus, the ECM plays an important role in the generation of apico-basal polarity in simple epithelial cells, and at the same time controls development of simple epithelial structures by orienting the mitotic spindle and cell division.

The unique liver architecture

The concepts and design principles of simple epithelia have now been briefly introduced. Though most of the tubular systems in the mammalian organisms are of the simple epithelial type, some organs have developed specialized tubular systems to serve their unique function and architecture. One of these organs is the liver. The liver is the largest metabolic organ. It is responsible for the generation of bile acids

and salts, storage of glycogen, iron, vitamins, and minerals, cholesterol homeostasis, plasma protein production, detoxification of the blood, and hormone and cytokine production [19,86]. The liver houses many cell types including endothelial cells, Kupffer cells (macrophages), pit cells (natural killer cells), hepatic stellate cells, and epithelial cells: cholangiocytes and hepatocytes. Hepatocytes constitute most of the liver cell mass (~78-85%), and provide most liver functions [87,88]. The general unit of the liver is the liver lobule (Figure 5A). The liver lobule can be considered as a hexagonal-shaped structure with in its center a central vein. From the central vein originate cords of hepatocytes that are lined with sinusoidal capillaries. At the edges of the lobule is a portal triad of vessels that include the portal vein, hepatic arterioles, and bile ducts. Nutrient-rich blood flows from the portal vein, together with oxygen-rich blood from the arterioles, to the central vein via the endothelial-cell lined sinusoids. This unique tissue architecture makes it very efficient for the liver to perform its functions. Hepatocytes generate bile in which they secrete toxic metabolites, but also bile salts that aid fat digestion. Excess bile is toxic for cells and thus the liver houses a complex network of canals and tubules, known as the biliary tree, to drain bile from the liver into the gall bladder and, ultimately, the intestine.

The biliary tree is composed of two distinct tubular systems and epithelial cell types

The biliary tree contains two morphologically distinct tubular networks that are each developed by two different epithelial cell types. The larger tubes of the biliary tree are located in the periportal area of the liver and are lined by cholangiocytes (Figure 5A and 5B, bile duct). Cholangiocytes are epithelial cells that exhibit simple epithelial apical-basal polarity and are aligned in monolayers that wrap around a bile-containing lumen, thus forming tube or duct-like structures. Cholangiocytes show typical simple (cuboidal) epithelial morphology and, like simple epithelium, sit on a basement membrane (cf. Figure 2). Hepatocytes, however, create a fundamentally distinct tubular or canalicular network (Figure 5B, bile canaliculus). In adult livers, and in striking contrast to the tubular network created by cholangiocytes, bile canaliculi are typically very small and often completely circumvent entire hepatocytes. Notably, the canaliculi form an anastomosing network that spans the entire liver parenchyma and drains bile from the hepatocytes into the bile ducts via a structure called the Canal of Hering (an intermediate canalicular-ductular structure).

It is intriguing how two fundamentally distinct tubular systems and cell types are generated in the same organ. Both epithelial cell types have their origin in the early

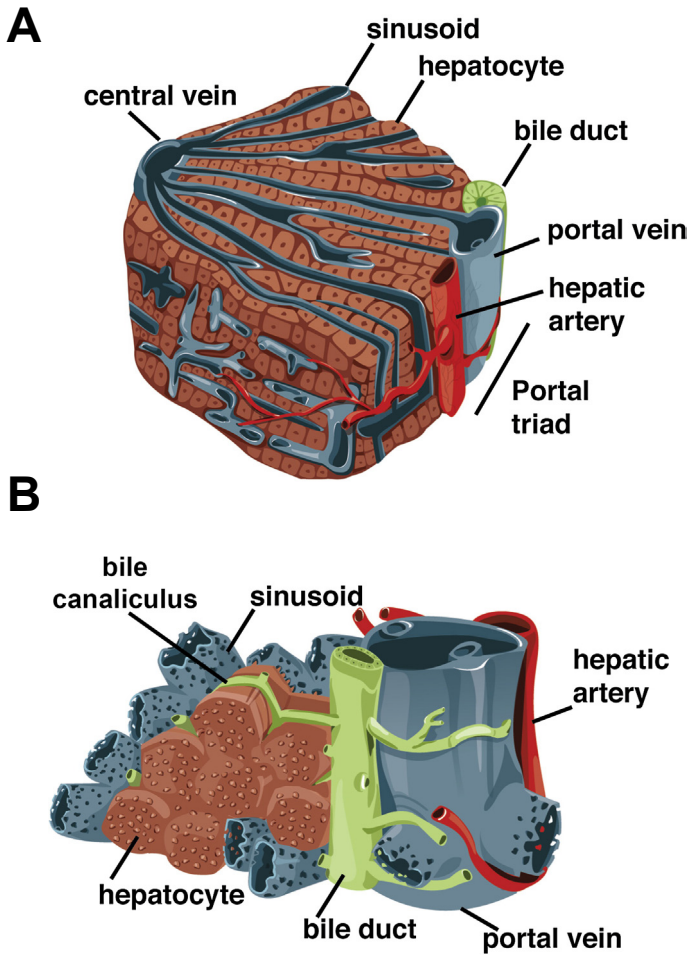

Figure 5. Organization of the liver parenchyma. **(A)** Architecture of the liver lobule. Blood flows from the portal vein and hepatic arteries to the central vein through the sinusoids. Hepatocytes aligned in cords exchange molecules via the sinusoidal capillaries. **(B)** Architecture of the hepatocytes and the bile canalicular network. Hepatocytes form a bile canalicular network that collects the bile produced by the hepatocytes. Bile flows from the bile canaliculi into the bile ducts, and ultimately into the gall bladder and intestine. Reproduced with permission from Elsevier from [87], © 2010.

stages of the developing liver and their development has been reviewed recently [19,87,89,90]. The liver's roots lie in the endoderm, more specifically at the site of the foregut which is also the origin of the pancreas, gall bladder, and lungs. Activation of factors including FoxA, GATA transcription factor, fibroblast growth factor (FGF), bone morhogenetic protein (BMP), Wnt-signaling, and hepatocyte nuclear factor (HNF) 1β and 6 promote liver-specific gene transcription and liver progenitor cell specification and proliferation of the initial liver mass. At ~E11 (embryonic day 11), a structure

comprised of hepatoblasts known as the liver bud is formed under the control of the T box transcriptional repressor Tbx3 and/or Prox1 [87]. Hepatoblasts are cells committed to forming liver-specific epithelial cells, and have the capacity to develop into both cholangiocytes and hepatocytes. At E13.5 in mice (7 weeks in humans), cholangiocyte specification of hepatoblasts is induced by transcription factors such as Sox9 and are first apparent in a structure called the ductal plate, which is comprised of a monolayer of cells that surrounds the periportal mesenchyme. During later stages and under the control of complex transcription factor signaling pathways that include factors such as TGF-β, Notch, Wnt, FGF, and BMP, a select amount of ductal plate cells ultimately develop into bile ducts (via a transient structure that is composed of both ductal plate cholangiocytes and hepatoblasts). Hepatocyte-specification involves transcription factors such as HNF4-α, HGF, C/EBPα, and absence of HNF1β, HNF6, and Wnt-signaling, though hepatocyte-specification has been difficult to investigate because it is not clear when exactly the differentiation program of hepatocytes is initiated. It is important to note that, during development of the liver, cholangiocytes and hepatocytes each occupy a spatially different and restricted niche within the liver tissue, and that their development and specification is regulated by a vast and complicated network of transcription factors.

Hepatocytes show a distinct polarity phenotype

As described earlier, the cholangiocytes and hepatocytes each constitute a distinct tubular phenotype. For simple epithelial cells (and, thus, cholangiocytes), the polarized architecture of individual cells (i.e., apical-basal polarity) is intimately linked to the 3-dimensional tubular architecture they develop. Indeed, hepatocytes, which show a distinct tubular architecture compared to cholangiocytes also exhibit a distinct type of cell polarity. In the healthy adult liver, hepatocytes are aligned in one or two-cell thick cords (Figures 5 and 6A). Hepatocytes have opposing basal surfaces that are in contact with the ECM and blood via highly permeable, endothelial-lined sinusoids (also known as the space of Disse), while connected to neighboring hepatocytes via their lateral membranes (Figure 6A). At the lateral membrane, hepatocytes form a small apical domain enclosed by tight junctions, that in the healthy adult liver constitutes the anastomosing bile canalicular network (Figure 6B). Even though the apical membranes are relatively small, they account for ~13% of the total hepatocyte surface area because of the extensive microvilli [91,92]. The distinct cell polarity phenotype of hepatocytes is better known as 'hepatocyte polarity'. Hepatocyte polarity is already apparent in the developing liver. At ~E12-13 in rats, adhering hepatocytes can be observed with small

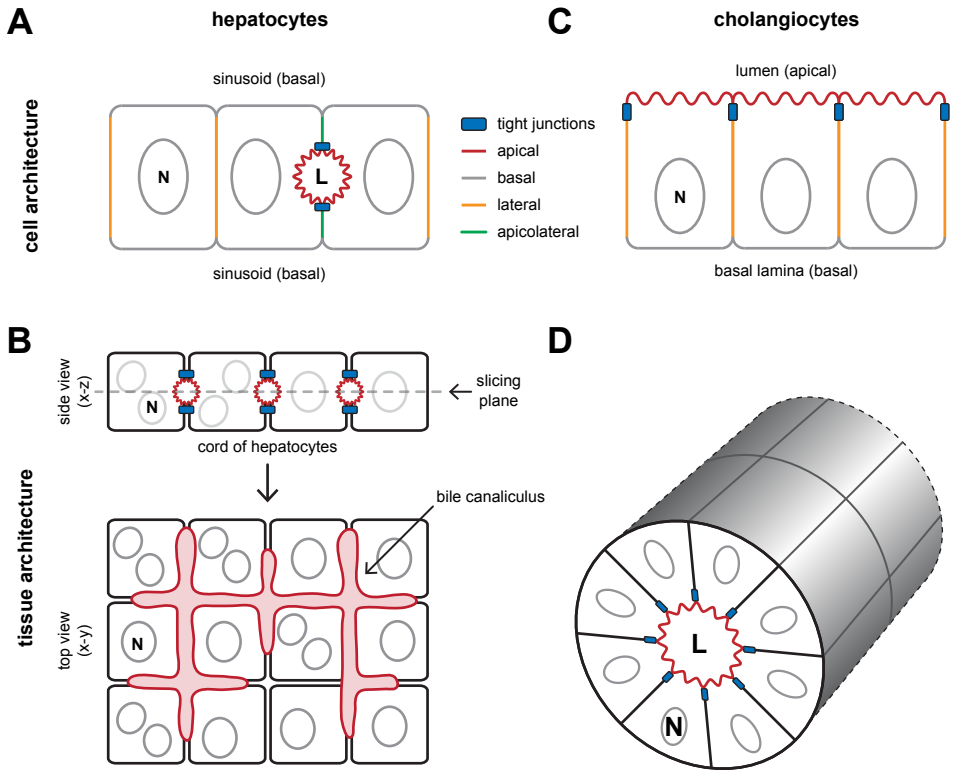

Figure 6. Hepatocytes have a distinct cell polarity and tubular phenotype compared to simple epithelial cells such as cholangiocytes. **(A)** The polarized architecture of hepatocytes is distinct from other epithelia. Hepatocytes form an apical lumen enclosed between two adjacent hepatocytes. Hepatocytes contact the blood and ECM via opposing basal plasma membrane domains. **(B)** Hepatocytes generate a distinct tubular system: the bile canalicular network. The canaliculi form an anastomosing network of canals throughout the liver parenchyma. **(C)** Polarized architecture of cholangiocytes and simple epithelial cells. **(D)** The tubular architecture generated by polarized cholangiocytes and simple epithelial cells. L = lumen. N = nucleus.

pre-canalicular pockets (i.e., lumens) observed at sites of attachment using electron microscopy [90,91,101]. Later (~E17), hepatocytes increase in number and appear to form more extensive cell-cell contacts, and first signs of elongated apical pockets appear [90]. Notably, multiple pre-canalicular lumens appear to have merged or fused together to form a cell-circumventing bile canalicular network. At E21, typical bile canalicular structures are observed and continue to develop postnatally [94,102]. Thus, the liver contains two distinct tubular networks that are each formed by distinct epithelial cell types. The distinct architecture of the tubular (canalicular) network formed by hepatocytes can be attributed to a unique type of cell polarity displayed by hepatocytes.

Hepatocyte polarity models

Various mammalian models have been developed to understand the development and molecular principles underlying hepatocyte polarity. The liver has a remarkable capacity to regenerate itself after injury. After hepatectomy, when up to 70% of the liver mass is removed, the liver initiates a regeneration program to replenish the liver mass to its original size. In otherwise healthy liver, it are mostly hepatocytes that start to proliferate and refill the liver, although there are reports of other hepatocyte cell sources such as hepatic stem or progenitor cells, and even cholangiocytes, via processes like de-differentiation/re-differentiation or trans-differentiation. Many of the pathways involved in liver development are active during liver regeneration as well [93–96]. Thus, liver regeneration serves as a suitable in vivo model to investigate formation of distinct tubular systems and cell polarity types in the liver.

Various in vitro cell models have also been developed [19,97]. Isolated primary hepatocytes are able to repolarize within 24 hours after plating (cf. Chapter 3) and retain polarity for multiple days, or weeks when plated in sandwich configuration [19,98]. A feature unique to primary hepatocytes is their ability to (after 3-7 days in culture) form extensive bile canaliculi [99,100]. However, they are not mitotically active and difficult to manipulate, rendering them unsuitable for studying the early phases of polarity development in the proliferating liver mass. Of course, experiments with these cells require a continuous supply of fresh livers for isolation, and are therefore time consuming and costly. Other cell models include the popular hepatoma HepG2 [101] cell line and the human-rat hybrid cell line WIFB9 [102]. These cell lines are able to go through developmental stages of hepatocyte polarity that include the formation of apical lumens at the lateral surfaces whilst they are still proliferating. Hence, these cell lines are ideally suited for studying apical membrane biogenesis and (pre-canalicular) lumen formation, aided by the fact that they are relatively fast-growing (HepG2 being superior in this case) and relatively easy to manipulate. HepaRG [103] cells are becoming increasingly popular as they are able to differentiate to both cholangiocyte and hepatocyte-like cells while retaining protein expression profiles of adult liver cells, making them suitable for differentiation and toxicity studies. However, culture conditions are much more difficult compared to HepG2/WIFB9, they grow relatively slow (2-4 weeks to generate hepatocytes), and their polarizing capacity remains to be investigated. Other cell lines, including WIFB derivates Huh7, Can 3-1, and Can 10 are also suitable for studying hepatic polarity, though they are less common [19,97]. Thus, a variety of models are available to study hepatocyte polarity.

Features and regulation of hepatocyte cell polarity

By using the cell models described above, some of the intracellular mechanisms and features of hepatocyte type polarity have been elucidated (reviewed in ref. [19]). One of the most striking features of hepatocyte polarity is the way proteins and lipids are sorted to their apical and basal plasma membrane domains. Whereas many apically destined proteins are directly targeted from the Golgi network to the apical plasma membrane in simple epithelial cells, these are indirectly (i.e., first to the basolateral, then to the apical plasma membrane domain; better known as transcytosis) transported in hepatocytes [19,104,105]. This is especially the case for single membrane-spanning and GPI-anchored membrane proteins, whereas polytopic apical proteins such as ABCB1/MDR1, ABCC2/MRP2, and ABCB11/BSEP are targeted directly to the apical membrane in both simple and hepatic epithelial cells [106]. In fact, secretion of proteins predominantly happens at the sinusoidal membrane in general in hepatocytes, in contrast to simple epithelial cells that target secretory components predominantly to the apical surface. The distinct intracellular trafficking routes of hepatocytes may partly be explained by the different expression of proteins that control sorting of proteins into the direct and indirect trafficking pathways. One of these proteins is the lipid-raft regulating myelin and lymphocyte protein (MAL) that in concert with MAL2 regulates trafficking of proteins towards the apical and basolateral surfaces, respectively. MAL is not expressed in hepatocytes, and exogenous expression of MAL in WIFB9 cells reroutes a subset of apical proteins back into the direct pathway [107–109]. Clathrin adapter proteins AP-1A and AP-1B regulate sorting of basolaterally destined cargo in epithelial cells [110–114]. AP-1A promotes direct trafficking of proteins from the trans Golgi network to the basolateral surface, while AP-1B promotes trafficking of basolateral-destined cargo through an intermediate endosomal compartment (known as the common recycling endosome). Interestingly, hepatocytes and WIFB cells lack AP-1B but still transport cargo through an intermediate endosomal compartment, further indicating that hepatocytes have evolved distinct intracellular sorting and trafficking machineries ([114] and references herein). Together these observations show that hepatocyte polarity correlates with distinct modes of intracellular sorting and trafficking.

Other research has focused on the mechanisms that stimulate or induce hepatocyte polarity. For example, studies in primary hepatocytes have elucidated an important role for bile acids in stimulating hepatocyte polarity and speeding-up bile canalicular network formation via pathways involving cAMP, Epac, LKB1/Par5, and AMPK [99,100,115,116]. Other work has shown that oncostatin M stimulates hepatocyte polarity via PKA in a cAMP-independent manner [117–119], indicating that multiple pathways exist to

stimulate hepatocyte polarity. A factor that is crucial for inducing hepatocyte polarity during liver development is HNF4α, and liver-specific knockout of HNF4α results in loss of hepatocyte polarity and hepatocyte tissue architecture ([87] and references herein), indicating that HNF4α (and the transcriptional network is regulates) is one of the few factors currently known that is absolutely necessary for development of hepatocyte polarity. HNF4α may thus serve as a molecular switch that determines adoption of either cholangiocyte (simple) or hepatocyte polarity in developing liver cells (hepatoblasts). Another factors that induces hepatocyte polarity is Par1b, as in vitro overexpression of the polarity protein EMK1/Par1b in MDCK cells induces a switch from simple epithelial polarity to hepatocyte polarity that includes the formation of lateral hepatocyte-like lumens. Coincidentally, Par1b overexpression also induces a hepatocytic (indirect) mode of apical (e.g., gp135/podocalyxin) protein trafficking [120–122], further strengthening the assumption that intracellular trafficking and polarity phenotypes are intimately linked. Importantly, the HNF4α and Par1b experiments indicate that molecular regulators exist that are able to control the adoption of either simple or hepatocyte type of polarity. These molecules may be of considerable interest for investigating how hepatocyte polarity is developed and maintained.

Hepatocyte polarity and lumenogenesis

As described earlier, individual simple epithelial cells use their apical-basal polarity axis as an internal guidance system to control mechanisms such as intracellular trafficking and mitotic spindle orientation in order to form a single central lumen and, ultimately, tubes. Notably, these mechanisms all share the same purpose to generate a single apical plasma membrane domain or lumen that is surrounded by a single-cell thick sheet of epithelial cells. An important extrinsic factor in regulating apical-basal polarity and its downstream lumen-forming mechanisms is the microenvironment (see paragraph "The microenvironment controls simple epithelial architecture"). The biliary tree of the liver consists of two distinct tubular systems and epithelial cell types (Figure 6). Cholangiocytes are simple epithelial cells with simple apical-basal polarity and form tubes that are typical for simple epithelial cells (i.e., a monolayer surrounding a central lumen) (Figure 6C and 6D). Hepatocytes, however, have a distinct form of apical-basal polarity which we call hepatocyte polarity (cf. Figure 6A), and form canaliculi circumventing entire cells (cf. Figure 6B). Importantly, the canaliculi are (from the canalicular membrane point-of-view) never shared by more than two cells. How hepatocytes use their distinct type of cell polarity to control lumen-forming mechanisms and form their canalicular network is not known.

Simple epithelial cells use symmetric orientation of cell division (coupled to the orientation of the mitotic spindle) and symmetric inheritance of plasma membrane domains for the development and maintenance of simple epithelial polarity and tubes. Indeed, mitotic spindle orientation also drives the formation of bile ducts [123]. However, these mechanisms are incompatible with formation of bile canaliculi, because symmetrical inheritance of apical plasma membrane domains in the developing liver would result in simple epithelial-like tubular systems, induce simple epithelial (cholangiocyte) type of polarity at the cost of hepatocyte polarity, and not allow formation of an anastomosing bile canalicular network. Thus, orientation of cell division is likely to be differently controlled in hepatocytes.

The specific composition of the microenvironment, especially the presence of basement membrane, is important for simple epithelial morphogenesis and controls apical-basal polarity formation and (symmetric) cell division orientation. Indeed, cholangiocytes sit on a basement membrane that controls the formation of simple epithelial polarity and bile ducts via basement membrane component laminin [76]. Hepatocytes occupy a spatially different niche and microenvironment. The space of Disse contains a different combination of ECM proteins and, importantly, lacks major basement membrane component laminin. Instead, the space of Disse contains a combination of mostly fibronectin, collagen type I, and minute amounts of collagen type III, IV, V, and VI [124–126], and facilitates the need for rapid transport of molecules between the blood and hepatocytes. Interestingly, during liver injury and fibrosis, ductal structures are known to develop outside of their normal (periportal) niche, and correlates with an altered microenvironment that includes deposition of basement membranes [124–126]. Importantly, these ductal structures can be formed by various hepatic cells, including hepatocytes [127–129]. Thus, the composition of the microenvironment is likely an important determinant and regulator in the formation of the two cell polarity types and tubular architectures in the liver. How the specific composition of the microenvironment regulates polarity and tubular phenotypes in the liver is not clear.

Another mechanism that simple epithelial cells use to form a single central lumen is the generation of a single apical domain or surface. During tube formation, simple epithelial cells constantly maintain a single apical surface to which secretion and apical protein trafficking is directed. Importantly, loss of factors that control the cell's commitment to only one apical domain, such as Slp2a, results in loss of simple epithelial polarity and tube integrity. Effectively, loss of Slp2a results in the formation of multiple apical domains per cell and is therefore incompatible with formation of a single central lumen.

How hepatocytes form lumens during liver development, and how they subsequently form a canalicular network, is unclear. Earlier work in fixed liver tissue sections suggest that hepatocytes form small apical lumens interspersed throughout the liver cell mass that later grow out and fuse together to form the bile canalicular network. However, the dynamics of hepatocytic lumen formation in the proliferating liver mass are unknown. Also, whether hepatocytes use apical lumen commitment in a similar way to simple epithelial cells (e.g., commitment to only one apical domain) during bile canalicular network formation, is not known.

Scope of this thesis

The central question that is addressed in this thesis is how the hepatocyte type of polarity is translated into lumen-forming mechanisms to develop and maintain hepatocyte polarity and, ultimately, the bile canalicular network. In **Chapter 2**, the dynamics and molecular mechanism of mitotic spindle and cell division orientation, and their role in the development and preservation of hepatocyte polarity, are studied in proliferating hepatocytes. Specifically, the role of the polarity protein Par1b (that, as described earlier, controls the developmental 'decision' to form simple epithelial or hepatocyte polarity) in mitotic spindle orientation and hepatocyte polarity is investigated. In **Chapter 3**, the effect of the microenvironment composition on hepatocyte lumenogenesis is studied in proliferating hepatocytes. Additionally, the involvement of the microenvironment on cell division orientation (and, thus, the development and preservation of hepatocyte polarity) is investigated. In **Chapter 4**, the lumen-forming dynamics in proliferating fetal-like hepatocytes are investigated, with an additional focus on factors such as Slp2a that control the cellular commitment to a single apical domain.

CHAPTER 2

Par1b induces asymmetric inheritance of plasma membrane domains via LGN-dependent mitotic spindle orientation in proliferating hepatocytes

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ABSTRACT

The development and maintenance of polarized epithelial tissue requires a tightly controlled orientation of mitotic cell division relative to the apical polarity axis. Hepatocytes display a unique polarized architecture. We demonstrate that mitotic hepatocytes asymmetrically segregate their apical plasma membrane domain to the nascent daughter cells. The non-polarized nascent daughter cell can form a de novo apical domain with its new neighbor. This asymmetric segregation of apical domains is facilitated by a geometrically distinct "apicolateral" subdomain of the lateral surface present in hepatocytes. The polarity protein partitioning-defective 1/microtubule-affinity regulating kinase 2 (Par1b/MARK2) translates this positional landmark to cortical polarity by promoting the apicolateral accumulation of Leu-Gly-Asn repeat-enriched protein (LGN) and the capture of nuclear mitotic apparatus protein (NuMA)–positive astral microtubules to orientate the mitotic spindle. Proliferating hepatocytes thus display an asymmetric inheritance of their apical domains via a mechanism that involves Par1b and LGN, which we postulate serves the unique tissue architecture of the developing liver parenchyma.

INTRODUCTION

The liver is a vital organ. Hepatocytes occupy more than 85% of the parenchymal liver mass and are responsible for a wide range of biological processes. These include the synthesis of plasma proteins and the processing of nutrients and toxic compounds from the blood that passes through the liver sinusoids. Hepatocytes also produce and secrete bile. Bile contributes to fat emulsion in the intestine and the elimination of detoxified compounds via the feces. Hepatocytes form a branching network of bile canaliculi between the cells that efficiently drains the secreted bile out of the liver parenchyme while keeping it separate from the blood [99,100]. The microanatomy of this canalicular network is unique to the liver [19]. Defects in the bile canalicular network and bile flow are associated with liver diseases [131].

Knowledge of the cell biological principles and molecular mechanisms that underlie the development of the bile canalicular network is limited. This is in part due to the lack of in vitro cell culture model systems that combine cell proliferation and canalicular network formation. Nevertheless, different in vitro cell model systems can reproduce specific steps in the process of bile canalicular network formation. For instance, from early microscopy studies of embryonic rat livers we know that the formation of isolated small spherical lumens between mitotically active hepatocytes is the first step in bile canalicular network development [132–135] (Figure 1A), and this process is reproduced by hepatic HepG2 [9,136] and WIF-B9 [102] cell lines. Both in vivo and in vitro, the formation of these primordial intercellular lumens is accompanied by the segregation of the hepatocyte surface into a lumen-facing apical domain and a sinusoid-facing basal domain, each with a specific protein and lipid composition (Figure 1A) [9,102,132,135,136]. The establishment of cell surface domains is the hallmark of apical–basal cell polarity [11].

The early establishment of apical–basal polarity is instrumental for the functional shaping of a proliferating epithelial cell mass [30,56]. Indeed, dividing cells not only generate enough critical cell mass to create the tissue, but they also make use of their apical–basal polarity axis (PA) to orientate their mitotic spindle apparatus [28]. By orientating its mitotic spindle apparatus, the dividing polarized epithelial cell can control the position of the emerging new nuclei, and hence the position of the daughter cells, relative to the position of the primordial apical domain and lumen. The same principles are used when dividing cells repair tissue damage [137].

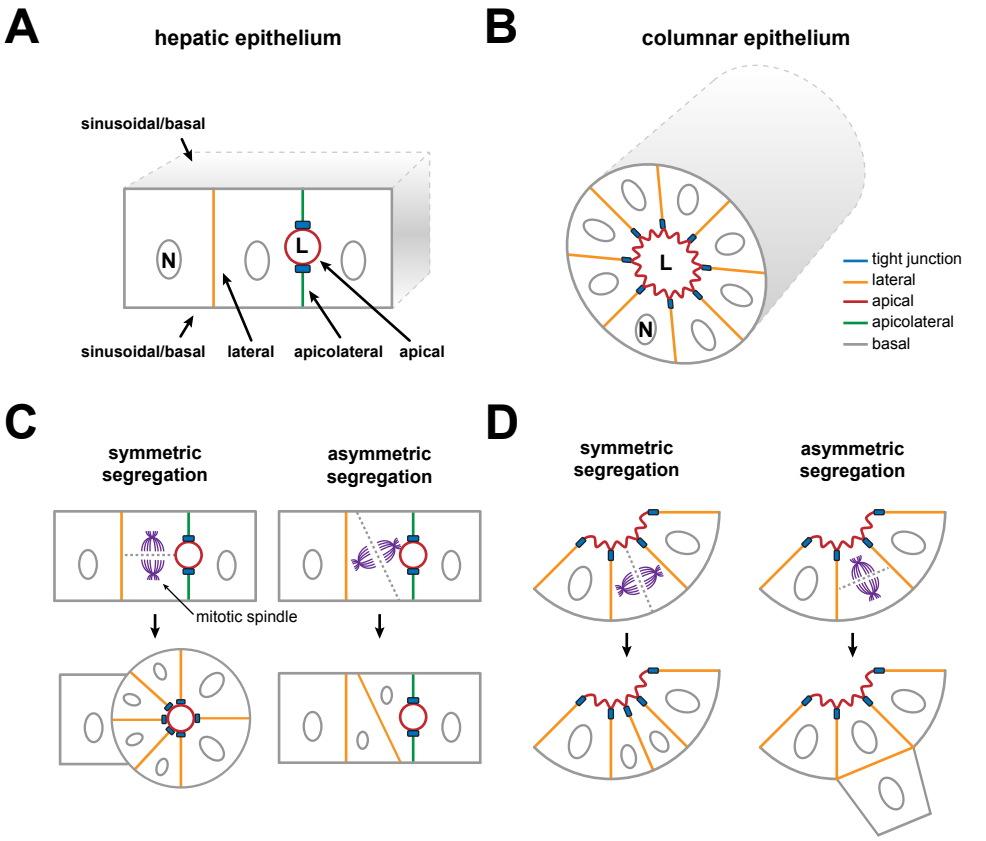


Figure 1. Schematic overview of the difference between columnar and hepatic epithelium. **(A)** Schematic overview of hepatic epithelium and polarity. Note the presence of an apicolateral plasma membrane domain in this type of epithelium. **(B)** Schematic overview of columnar (i.e., "simple") epithelium and polarity. **(C)** Schematic representation of the tissue architecture resulting from symmetric and asymmetric segregation of the apical surface in hepatic epithelium. **(D)** Schematic representation of the tissue architecture resulting from symmetric and asymmetric segregation of the apical surface in columnar epithelium. The dashed line represents the cleavage furrow and the position of the newly formed membrane. L, lumen; N, nucleus.

The unique microanatomy of the bile canalicular network suggests that the mode of cell division orientation in hepatocytes—from the moment that they have established apical–basal polarity—differs from that observed in "simple" epithelial cells such as intestinal or kidney epithelial cells. Indeed, simple epithelial cells do not develop a canalicular network between cells. Instead, they develop large cystic lumens and tubes (Figure 1B) via a process that is dependent on a Leu-Gly-Asn repeat-enriched protein (LGN)–mediated orientation of the mitotic spindle apparatus that is strictly perpendicular to the apical–basal axis, and the resultant symmetric segregation of the apical domain to both daughter cells [66,69,72] (Figure 1D). A mitotic spindle orientation that

is strictly perpendicular to the apical PA would in mitotic hepatocytes thus be predicted to promote the development of cystic lumens rather than of canalicular networks (Figure 1C).

In order to investigate the orientation of the mitotic spindle in hepatocytes within their native environment, immunohistochemistry can be performed on fixed liver tissues. To study the molecular regulation and the dynamics of mitotic spindle orientation and cell division in hepatocytes, cell lines are the best model of choice. In this study we have combined the analysis of liver tissues with that of HepG2 and WIF-B9 cell lines to investigate the relationship between cell polarity and the orientations of the mitotic spindle and cell division at the molecular level in hepatocytes.

MATERIALS AND METHODS

Antibodies and reagents

Commercial antibodies used for immunofluorescence are listed in Table S1. The rabbit anti-LGN antibody was described earlier [51]. Phalloidin-TRITC was used to label F-actin (P1951; Sigma). DAPI was from Invitrogen, and DRAQ5 was purchased from Cell Signaling Technology.

Plasmids

The plasmid expressing H2B-mCherry was a kind gift from B. Giepmans (University Medical Center Groningen, the Netherlands). The plasmid expressing human Par1b was a kind gift from H. Miki (Osaka University, Japan). Overexpression was obtained by cloning constructs into a lentiviral expression system [138]. Briefly, constructs were cloned into pENTR1A (Addgene plasmid 17398) and recombined into pLenti-CMV-Puro (Addgene plasmid 17452) using LR clonase (Life Technologies). The Par1b-KD construct was described before [120].

Cell lines and tissues

HepG2 cells were cultured as previously described [139]. HepG2 cells expressing ABCB1-eGFP were cultured as previously described [7]. For experiments, cells were plated on ethanol-sterilized glass coverslips at a density of 5×10^4 cells/cm^2 and grown for 2 d. For Par1b knockdown experiments, cells were plated at 15×10^4 cells/cm^2 to match WIF-B9 conditions (see below). WIF-B9 cells were grown in modified F-12 Coon's

modification medium (F6636; Sigma) supplemented with 10^{-6} M hypoxanthine, 4×10^{-8} M aminopterin, 1.6×10^{-6} M thymidine, 5% (v/v) fetal bovine serum (100–106; Gemini), 1% glutamax (Invitrogen), 0.5 μg/ml amphotericin, and 10 mM HEPES. For culture maintenance, cells were seeded in plastic dishes at 10×10^{3} cells/cm^2 and cultivated up to 4 d before replating. For experiments, differentiated cultures (10–12 d) were plated on water-prewashed glass coverslips (EMS) in MatTek chambers at 15×104 cells/cm2. Madin-Darby canine kidney (MDCK) cells were grown in DMEM without phenol red (17–205; Cellgro) supplemented with 10% fetal bovine serum (S11050; Atlanta Biologicals) and 2 mM L-glutamine. Stable MDCK cell lines expressing gp135-GFP and Par1b were generated from T23-MDCK cells. MDCK-Par1b cells were prepared as previously described [120]. Cells were maintained at 37°C in a 5% CO^2 (HepG2 and MDCK cells) or 7% CO^2 (WIF-B9 cells) humidified atmosphere. Mouse liver tissues 48 h after partial hepatectomy (formalin-fixed paraffin-embedded) and mouse liver tissue from 23-d-old mice, collected 2 d after weaning (prepared as near-native tissue slices as previously described [140]), were prepared as previously described [141]. Formalin-fixed paraffin-embedded rat liver tissue (collected 2 d after weaning) was a kind gift from C. Desdouets (INSERM, France).

RNA interference

For HepG2 cells, RNA interference was performed using the pLKO lentiviral knockdown system (http://www.addgene.org/tools/protocols/plko/). The target sequences used for Par1b and LGN are listed in Table S2 and were generated according to the pLKO protocol. Knockdown was verified by real-time PCR on a StepOnePlus system (Applied Biosystems) using the primers listed in Table S3. A short hairpin RNA (shRNA)–resistant Par1b was created by introducing missense mutations into the shRNA target sequence (AGCAAGAGAGGCACTTTA toAGTAAAAGGGGAACATTG) using a Q5 Site-Directed Mutagenesis Kit (E0554S; New England Biolabs). All constructs were verified by sequencing. RNA was isolated using Tri-Reagent from Sigma (T9424). RNA interference experiments in WIF-B9 and MDCK cells were performed as previously described [120].

Virus production and transduction

Lentiviral particles were created using a second-generation system based on pCMVdR8.1 (structural components) and pVSV-G (envelope protein). Briefly, 2.6×10^{6} HEK293T cells were plated in a 10-cm dish. The next day, the cells were co-transfected with CaPO4-DNA complexes of pCMVdR8.1, pVSV-G, and either pLKO.1 or pLenti constructs for ~16 h. Medium was refreshed, and after 24 and 48 h viral particles were harvested and filtered

through a 0.45-µm PVDF membrane filter. Viral supernatants were stored at −80°C. 1-d-old HepG2 cells were infected with viral particles for 24 h, whereafter cells were incubated with normal growth medium to recover from the viral infection. Selection medium (1 µg/ml puromycin; Sigma) was added 24 h later to select for transduced cells. WIF-B9 cells expressing DPPIV-TagRFP, GFP, Par1b-DN-GFP, pSUPER-GFP, or shRNA Par1b-GFP were obtained by adenovirus-mediated transduction [120] in Opti-MEM (Invitrogen) for 1 h with one plaque-forming unit/cell and 10–12 h expression at 37°C.

Immunofluorescence and microscopy

Cells were fixed in 4% paraformaldehyde at 37°C (MDCK and WIF-B9 cells) or for 20 min at room temperature (HepG2 cells). For staining microtubular structures in HepG2 cells (tubulin, NuMA, and LGN), cells were pre-extracted in 0.5% Triton X-100 in PHEM buffer (1 min), washed once in PHEM buffer, and fixed in 4% paraformaldehyde in PHEM buffer. For HepG2 cells, blocking and permeabilization were performed for 30 min at room temperature in HBSS containing 0.025% saponin (w/v), 1% (w/v) BSA, and 0.02% sodium azide, followed by antibody staining in the same buffer. WIF-B9 and MDCK cells were permeabilized with 0.2% Triton X-100 and blocked with 1% BSA. Antibody incubation was performed in PBS–1% BSA. HepG2 cells were imaged using a combination of widefield (Olympus AX70) and confocal microscopy (Leica SP2; HCX PL APO 63x/1,4 oil; pinhole 1 AU; pixel size 80 nm) and analyzed using a combination of Imaris (Bitplane), ImageJ, and Adobe Photoshop CS4. A Solamere Nipkow confocal live cell imaging system was used (HCX PL APO 63x/1,3 glycerin; pixel size 117 nm) to live-image z-stacks of 7×1.5 µm every 4 min, unless otherwise indicated. WIF-B9/MDCK cells were imaged with a TCS SP5 confocal microscope (Leica Microsystems) equipped with a motorized x-y stage for multiple position finding and with an 8,000-Hz resonant scanner. Fixed cells were imaged using a HCX PL APO 63x/1.4-0.60 oil λBL CS objective on glass coverslips mounted in non-hardening, glycerol-based aqueous mounting medium. Confocal (pinhole 1 AU; pixel size 80.02 nm) xyz-stacks were taken from the monolayer. Live cell imaging was performed using a HCX PL APO 40x/1.25-0.75 oil CS objective on MatTek or CELLview chambers. xyzt-stack frames (pinhole 2–3 AU; pixel size 100.1–252.8 nm) were recorded. Image analysis was performed using LAS AF 2.3.1 and ImageJ 1.45 software. Brightness and contrast were adjusted according to the Journal of Cell Biology guidelines, without changing gamma settings.

Calculations and statistics

For calculating the orientation of the mitotic spindle in cell lines, a line was drawn from the center of the apical lumen through the center of the mitotic spindle (PA). A second line was drawn through the spindle poles (spindle axis [SA]). When no spindle pole staining was performed, it was assumed that the spindle poles were localized in a straight line perpendicular to the metaphase plate. The angle between these lines (SA/PA) was calculated with the ImageJ measure angle tool and plotted accordingly. To study the orientation of cell division in rat and mouse liver tissue, a line was drawn through both spindle poles of a dividing cell and extrapolated to determine the plasma membrane domain to which the spindle poles were oriented. The orientation of the spindle poles was scored as oriented towards (1) the bile canaliculus, (2) the apicolateral domain, or (3) the basolateral (sinusoidal) or common lateral membrane. Microsoft Excel was used for calculations, and Graphpad PRISM was used to generate graphs. Graphs represent mean ± standard deviation of three independent experiments, unless otherwise specified. Sample sizes (n) in graphs represent the total sample size. The statistical significance of differences was determined using Student's t-test (two-tailed, unpaired, with equal variance) unless otherwise specified.

RESULTS

The mitotic spindle in hepatocytes in vivo is orientated towards an LGN-enriched apicolateral plasma membrane domain

We first analyzed the orientation of the mitotic spindle relative to the apical PA in vivo in mitotic mouse hepatocytes that were in metaphase or in telophase 48 h after hepatectomy. A line drawn through the mitotic spindles poles (immunolabeled with antibodies against the microtubule-binding nuclear mitotic apparatus protein [NuMA]) typically intersected the dipeptidyl peptidase 4 (DPPIV)–positive apical canalicular domains (Figure 2A, arrowheads) or its flanking regions, rather than the basolateral/sinusoidal domains (Figure 2A, sinusoidal domains are indicated by "si" and dotted lines). These data are in agreement with earlier observations in proliferating rat hepatocytes following hepatectomy [142,143]. Quantification of confocal images of 61 mitotic hepatocytes from three mice 48 h after hepatectomy (see Materials and Methods) revealed that 85.1%±10.1% of the mitotic spindle axes intersected the apical bile canalicular or apicolateral domain (Figure 2B). We also analyzed the orientation of the mitotic SA relative to the apical PA in hepatocytes in vivo in fixed liver tissue from young healthy mice (Figure S1A) and rats (Figure 2C and 2D), which display a

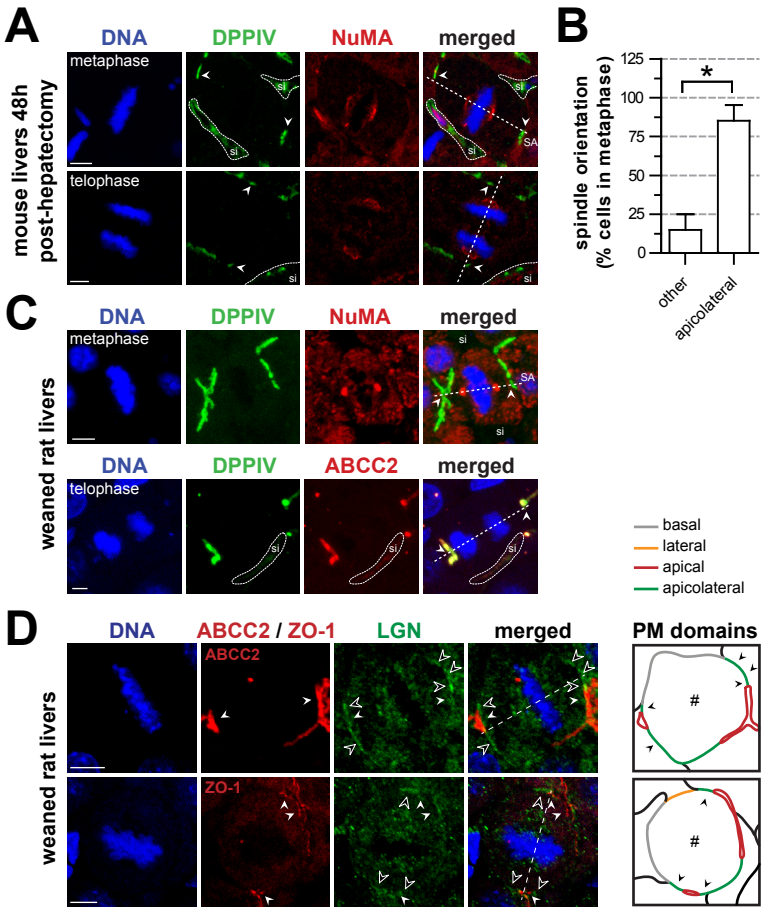


Figure 2. Rat and mouse hepatocytes predominantly orient their mitotic spindle axis towards the apicolateral subdomain. **(A)** Hepatocytes from mouse livers 48 h post-hepatectomy orient their spindle poles (labeled with NuMA) towards the apicolateral subdomain. **(B)** Quantification of (A) (n = 61). Dividing hepatocytes predominantly orient their SA towards the apicolateral subdomain. **(C)** Dividing hepatocytes from weaned rat livers orient their spindle poles (marked by NuMA) towards the apicolateral subdomain (marked by DPPIV and ABCC2) in metaphase and telophase. **(D)** Apicolateral localization of LGN (white outline arrowheads) in dividing rat liver hepatocytes. The apical domain is labeled with ABBC2. Tight junctions are labeled with ZO-1. The outline diagrams ("PM domains") show the identity of the cell membranes of the dividing cells (#) shown in (D). Grey, red, orange, and green lines represent the basal, apical, lateral, and apicolateral plasma membrane domains, respectively. All figures: filled white arrowheads mark the bile canaliculus or apical domain. Dotted white lines outline the sinusoid (si). Dashed lines indicate the SA. * $p < 0.05$ (calculated using a paired two-tailed Student's t-test). Scale bars: 5 μm.

burst of cell division after weaning [141,144]. A line drawn through the NuMA-labeled mitotic spindle poles of rat hepatocytes that were in metaphase or in telophase typically intersected the DPPIV-positive apical bile canalicular plasma membrane domain or its flanking region (Figure 2C). We named this flanking region the apicolateral domain, as it

could be distinguished from the "common" lateral plasma membrane facing neighboring cells with which no apical lumen was (yet) formed (Figures 2D and S1B; Movie S1), and is a geometrically distinctive feature of cells with a hepatic polarity phenotype (Figure 1A). The orientation of the mitotic spindle in hepatocytes in vivo correlated well with a restricted localization of the mitotic-spindle-orientating protein LGN [53,62,63,145] at and/or in close proximity to the zona occludens 1 (ZO-1)–marked tight junctions at this apicolateral region flanking the apical/bile canalicular domain (Figure 2D, green lines in the diagram). LGN was largely absent from the "common" lateral plasma membrane facing neighboring cells with which no apical lumen was shared and absent from basal/sinusoidal plasma membrane domains (Figure 2D, orange and grey lines, respectively, in the diagram). These data demonstrate that in hepatocytes in vivo, the mitotic spindle is predominantly orientated towards an LGN-enriched apical/apicolateral surface domain.

The typical orientation of the mitotic spindle to LGN-enriched apicolateral plasma membrane domains in hepatocytes in vivo is reproduced in cultured HepG2 cells

To investigate the dynamics of mitotic spindle orientation and cell division and its molecular regulation in living hepatocytes, we made use of the polarizing human hepatocyte cell line HepG2 [9,146]. HepG2 cells develop apical lumens amidst their lateral surfaces facing adjacent cells (Figures 1A and 3), reflecting the earliest stages of apical–basal polarity development in the fetal liver [132,134,135,139,147]. Importantly, in agreement with the observations in hepatocytes in vivo, a line drawn through the spindle poles in HepG2 cells in more than 70% of all cases intersected the apical or apicolateral domain (Figures 3A and S2A), where LGN was almost exclusively localized (Figure 3B). Note that LGN was not detected at the "common" lateral and sinusoidal plasma membrane domains (Figure 3B, apicolateral domains are green and "common" lateral and sinusoidal domains are orange and grey, respectively, in the diagram). To more accurately determine the orientation of the mitotic spindle relative to the apical PA in these cells, we measured the angle between the SA (the line drawn through the spindle poles; Figure 3C, dotted line) and the PA (the line drawn between the center of the immunolabeled apical domain and the center of the mitotic spindle; Figure 3C, solid line). 50.9%±7.0%, 32.1%±6.8%, and 17.0%±0.3% of the mitotic spindle axes in cells that were in metaphase displayed an SA/PA angle of 0–30°, 31–60°, and 61–90°, respectively, with statistically significant differences between the three categories (Figure 3D and 3E). A similarly biased SA/PA angle distribution was observed in cells that were in later stages of mitosis, i.e., anaphase or telophase (Figure S2A–S2C), and

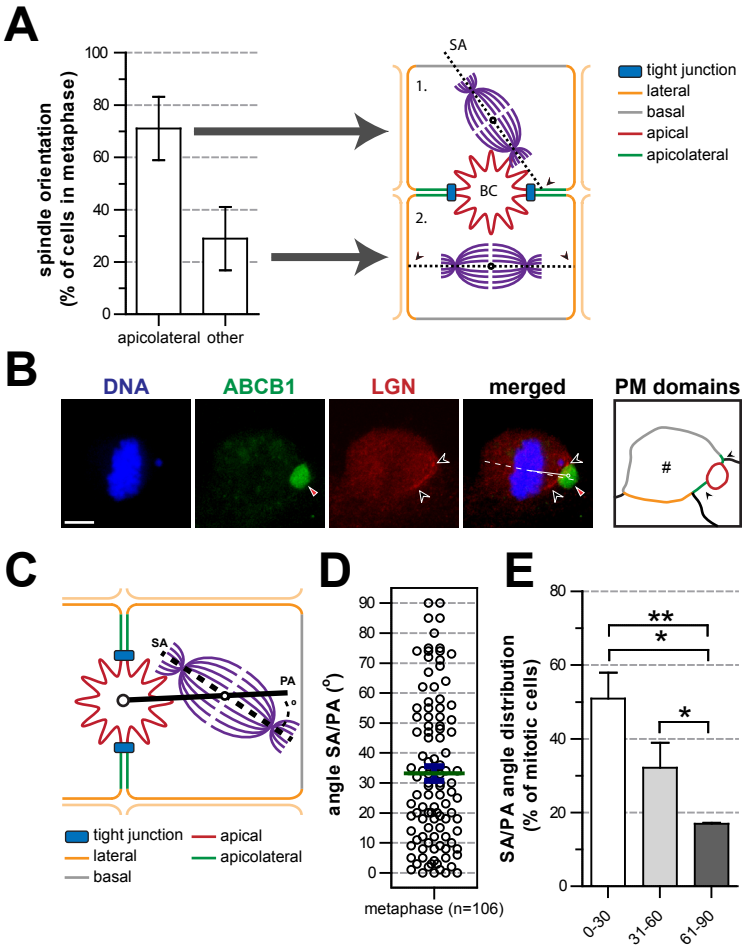


Figure 3. Hepatocytes predominantly orient their mitotic spindle axis towards the apicolateral subdomain. **(A)** SA axes were quantified as crossing (marked by black arrowheads) the apicolateral membrane (situation 1) or other membranes (situation 2), indicating a bias of the SA axis to cross the apicolateral membrane. **(B)** Localization of LGN (white outline arrowheads) in polarized HepG2 cells. The apical domain is labeled with ABCB1 and marked by a red arrowhead. The outline diagram ("PM domains") shows the identity of the cell membranes of the dividing cell (#). Grey, red, orange, and green lines represent the basal, apical, lateral, and apicolateral plasma membrane domains, respectively. **(C)** Schematic overview of how the orientation of the mitotic spindle (angle between the SA and PA [angle SA/PA]) was measured (see Materials and Methods). **(D)** Dot plot of the SA/PA angle for dividing HepG2 cells in metaphase. Shown is mean (green bar) and standard error of the mean (SEM) (blue error bars). **(E)** Histogram analysis reveals a strong bias for HepG2 cells to divide with an SA/PA angle between 0° and 30° during metaphase. * $p < 0.05$; ** $p < 0.01$. BC, bile canaliculus. Scale bars: 5 µm.

live cell imaging in HepG2 cells showed that the apicolateral-directed orientation of the mitotic spindle was fixed early in mitosis and remained stable throughout the subsequent mitotic stages (Figure S2D and S2E; Movie S2). The mitotic spindle did not show a

selective positioning or movement towards either spindle pole-facing domain of the cell (Figure S2F and S2G). These data demonstrate that HepG2 cells, similar to hepatocytes in vivo, orient their mitotic spindle with a significant bias towards an LGN-enriched apicolateral plasma membrane domain. These cells are therefore a useful model system to study the consequences of this stereotypic mitotic spindle orientation with regard to cell polarity and its molecular regulation.

Dividing HepG2 cells segregate their apical/bile canalicular plasma membrane domain asymmetrically to the two emerging daughter cells

Concomitant with the predominant apicolateral-plasma-membrane-directed orientation of the mitotic spindle apparatus, live cell imaging revealed that HepG2 cells predominantly divided in such a way that only one of the two emerging daughter cells inherited the apical lumen. Stills from a representative movie (Movie S3) of dividing HepG2 cells that express the green fluorescent eGFP-tagged apical protein ABCB1 (Figure 4A) show two non-mitotic (interphase) cells with ABCB1-eGFP-positive apical plasma membrane domains and lumens (indicated by the red arrowhead). After each cell passed through metaphase it formed a cleavage furrow (Figure 4A, black arrowheads) during anaphase/telophase that, following subsequent cytokinesis, gave rise to one daughter cell (marked by "1") that inherited the apical domain (red arrowhead) and one daughter cell (marked by "2") that did not. The vast majority of live cells (>75%) showed this asymmetric segregation of the apical plasma membrane domain and lumen during mitosis (Figure 4B). Similar results were observed with another hepatocyte cell line, WIF-B9 (Figure S3A and S3B; Movie S4), underscoring that this mode of cell division orientation is a feature of polarized hepatocytes and not only of HepG2 cells. Interestingly, we observed that the emerging non-polarized daughter cells could reestablish apical–basal polarity and reestablish an apical lumen with their new neighbor cells. An example of this is shown in the stills (Figure 4C) from Movie S5. These stills show that a HepG2 cell with an ABCB1-eGFP-positive apical plasma membrane domain and lumen (red arrowhead) formed a cleavage furrow (white arrowheads) that gave rise to one daughter cell (marked by the asterisk) that did not inherit the apical domain (red arrowhead), but reestablished an ABCB1-eGFP-positive apical domain (yellow arrowhead) with its sister at the site of cytokinesis. Taken together, we conclude that hepatocytes orientate their mitotic SA with a significant bias towards an LGN-enriched apicolateral plasma membrane domain, and asymmetrically segregate their apical plasma membrane domain and apical lumen to the emerging daughter cells.

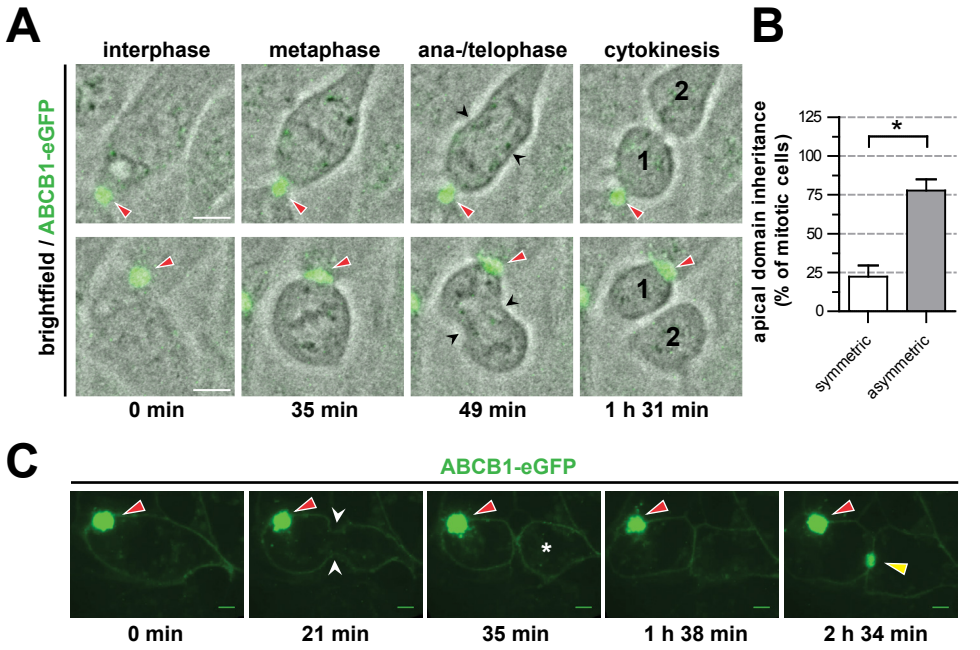


Figure 4. Hepatocytes segregate the apical plasma membrane and lumen asymmetrically during mitosis. **(A)** Stills from Movie S3 showing asymmetric segregation of the apical plasma membrane (ABCB1-eGFP, red arrowheads) in dividing HepG2 cells. Black arrowheads mark the ingressing cleavage furrow during cytokinesis. "1" marks the daughter cell inheriting the apical domain, and "2" marks the daughter cell not inheriting the apical domain, hence becoming non-polarized. **(B)** Quantification of the asymmetry of apical plasma membrane inheritance in dividing HepG2 cells (live imaging; n = 64). **(C)** Stills from Movie S5 showing asymmetric segregation of the apical domain (ABCB1-eGFP, red arrowheads) and formation of a new apical domain by the new daughter cell. White arrowheads mark the ingressing cleavage furrow. The yellow arrowhead marks the de novo formed apical domain at the site of cytokinesis. * $p < 0.05$. Scale bars: 5 µm.

Par1b overexpression in simple epithelial cells coordinates the adoption of a hepatic polarity phenotype with changes in the localization of LGN and mitotic spindle and cell division orientation

In order to investigate to what extent the hepatocyte polarity phenotype—as such—was sufficient to dictate an apicolateral-domain-directed orientation of the mitotic spindle, we made use of our earlier observation that the overexpression of the polarity protein partitioning-defective 1/microtubule-affinity regulating kinase 2 (Par1b/MARK2) in simple epithelial cells induces a hepatic polarity phenotype (schematically depicted in Figure 5A) [120]. Thus, when Par1b was overexpressed in MDCK cells (a widely used model of simple epithelial cells), apical plasma membrane proteins such as gp135 localized to lumens formed between adjacent cells (Figure 5B, Par1b), rather than to the cell-culture-medium-facing cell surface at the top of the control cell monolayer

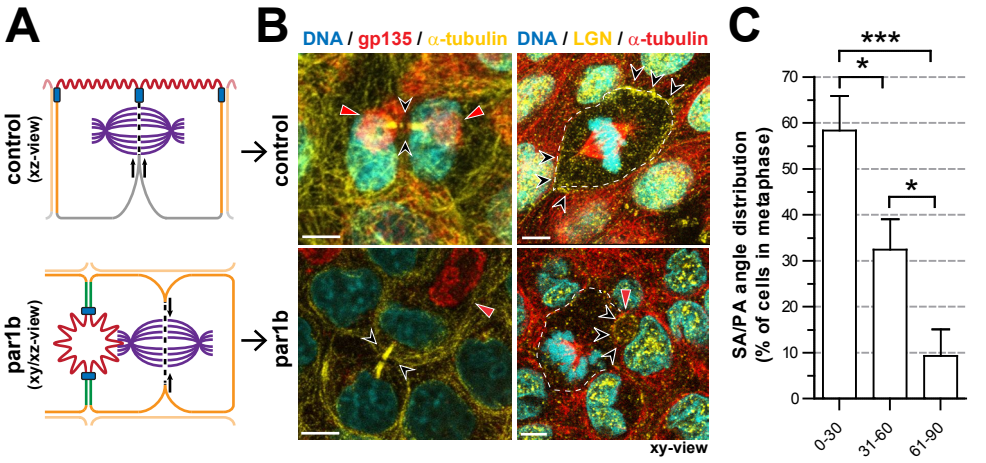


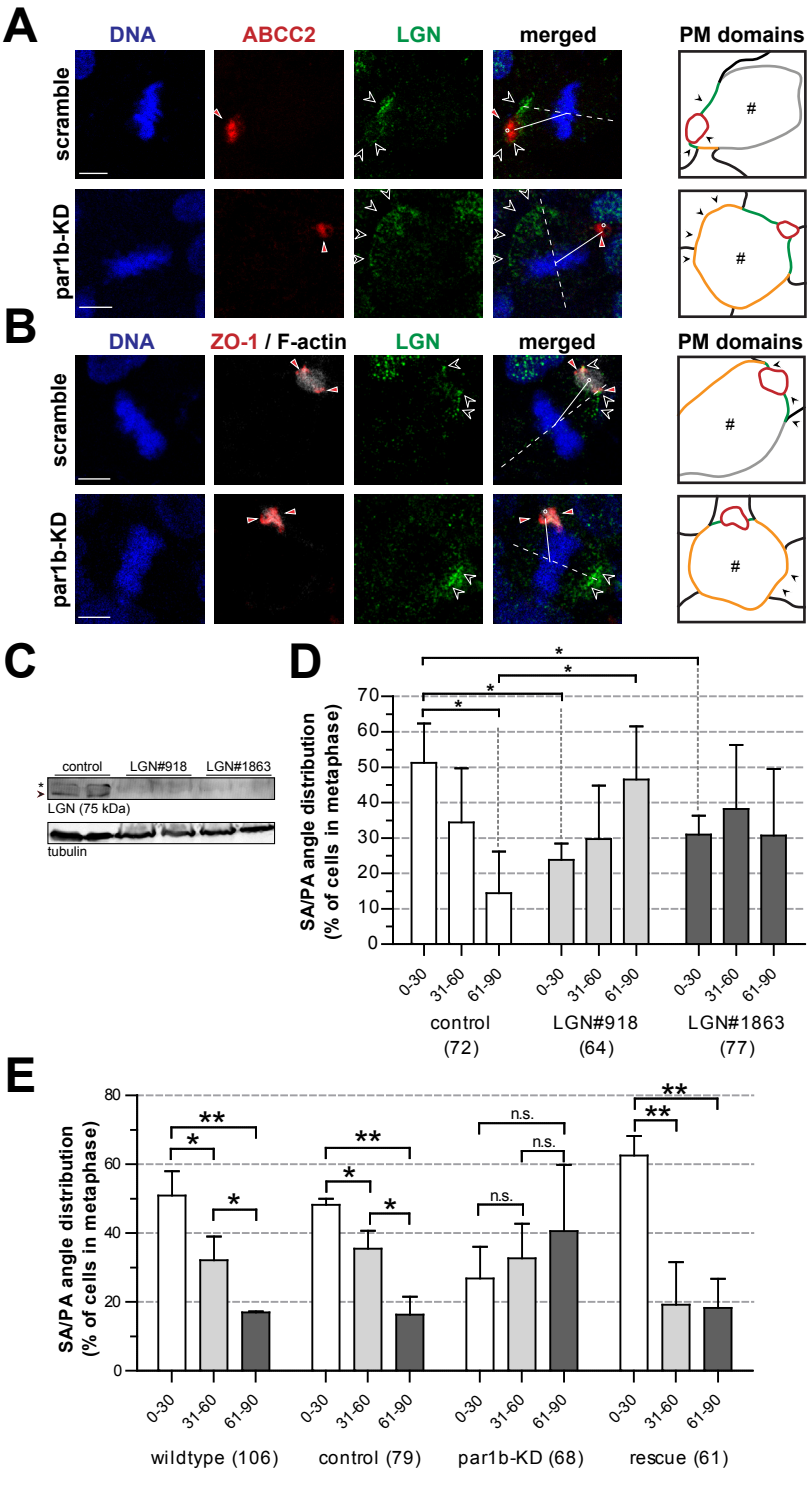
Figure 5. Par1b stimulates apicolateral-directed spindle orientation and asymmetric segregation of the apical domain in MDCK cells. **(A)** Schematic overview of the polarity phenotype in control and Par1b-overexpressing MDCK cells. **(B)** Fixed control and MDCK-Par1b cells were labeled for the apical marker gp135 (left panel, red) and LGN (right panel, yellow). Par1b-overexpressing MDCK cells asymmetrically segregate their apical domain (left panel, red arrowheads; black arrowheads mark the ingressing cleavage furrow) during cell division. LGN localizes to the apicolateral plasma membrane domain in Par1b-overexpressing MDCK cells (right panel, black arrowheads). The dashed line marks the common lateral plasma membrane domain. **(C)** Histogram analysis of the SA/PA angle shows that dividing MDCK-Par1b cells exhibit a bias towards lower angles (0–30°) in metaphase, as observed for hepatocytes. All figures: red arrowheads mark the apical domain. * $p < 0.05$. *** $p < 0.001$. Scale bars: 5 µm.

(Figure 5B, control). In mitotic cells identified in parental MDCK cell cultures, LGN was localized at the lateral plasma membrane domains and was excluded from the apical domain (Figure 5B, control, dotted white line; and previous reports [66]). In agreement with the role of cortical LGN in spindle orientation in these cells [66], 100% of all SA/PA angles have been demonstrated to be in the 61–90° range, giving rise to the symmetric segregation of apical and basal domains to both daughter cells [61,148]. In contrast, in MDCK cells that overexpressed Par1b and displayed a hepatic polarity phenotype, the localization of LGN was highly polarized and restricted to the apical/apicolateral domain (Figure 5B, Par1b, red arrowhead), and LGN was excluded from the "common" lateral cell surfaces (Figure 5B, Par1b, dotted white line). Coinciding with this change in the distribution of LGN, 58.3%±7.5%, 32.4%±6.6%, and only 9.3%±5.8% of all SA/PA angles were in the 0–30°, 31–60°, and 61–90° range, respectively, with a clear, statistically significant difference between these categories (Figure 5C). These data demonstrate that the overexpression of Par1b in simple epithelial cells caused a strong shift from a mitotic spindle orientation that was perpendicular to the apical PA to one that was significantly more parallel to the apical PA, matching a change in the distribution of LGN. Concomitantly, live cell imaging revealed that Par1b-overexpressing

MDCK cells, like hepatic cells, predominantly divided in such a way that only one of the two emerging daughter cells inherited the apical lumen (Figure S4A–S4C; Movie S6). These data show that Par1b coordinates the induction of a hepatic polarity phenotype with a change in (1) the localization of LGN, (2) the orientation of the mitotic spindle, and (3) the asymmetric segregation of the apical plasma membrane domain to the emerging daughter cells.

Par1b regulates mitotic spindle orientation by controlling the apicolateral enrichment of LGN in HepG2 cells

The results from the Par1b-overexpressing MDCK cells as displayed in Figure 5 do not demonstrate whether solely the induction of a hepatic polarity phenotype—as such—was sufficient to change the localization of LGN and the orientation of the mitotic spindle. Therefore, in order to further investigate to what extent Par1b is important for the localization of LGN and spindle orientation in the context of the hepatic polarity phenotype, we knocked down Par1b in HepG2 cells (Figure S5A). Confocal images in Figure 6A and 6B show that in control cells—i.e., cells treated with scrambled shRNA (scramble)—LGN (black arrowheads) localized at the cell surface flanking the ABCC2- or ZO-1/actin-labeled apical domain (red arrowheads), similarly to in untreated cells (cf. Figure 3B). In contrast, in cells treated with shRNA against Par1b (Par1b-KD), the localization of LGN was no longer restricted to the apicolateral domain (Figure 6A and 6B). LGN frequently showed an (additional) localization at the "common" lateral domain (black arrowheads, the "common" lateral domain is orange in the diagram), away from the apical domain (Figure 6A and 6B, red arrowhead). Note that these cells have retained the typical hepatic polarity phenotype. The change in the distribution profile of LGN was accompanied by a change in the orientation of NuMA-positive astral microtubules that emanated from the mitotic spindle poles and reached out to the cell cortex (z-stack sections inFigure S5B; Movies S7 and S8). The knockdown of LGN in HepG2 cells with two different shRNAs (Figures 6C and S6A) caused a randomization of the SA/PA angle in the 0–30°, 31–60°, and 61–90° categories, and caused a significant reduction of SA/PA angles in the 0–30° category when compared to control cells (Figures 6D, S6B, and S6C), hence underscoring the contribution of LGN to mitotic spindle orientation in these cells. In agreement with the change in LGN distribution, the knockdown of Par1b caused a randomization of the orientation of the mitotic spindle axes, with approximately 26.8%±9.2%, 32.6%±10.1%, and 40.6%±19.2% displaying an SA/PA angle between 0–30°, 31–60°, and 61–90°, respectively, with no statistically significant difference between the three categories (Figure 6E). Thus, Par1b


A
DNA
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B
DNA
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PM domains
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par1b-KD
#
C
control
LGN#918
LGN#1863
LGN (75 kDa)
tubulin
D
SA/PA angle distribution (% of cells in metaphase)
0
10
20
30
40
50
60
70
0-30
31-60
61-90
control (72)
LGN#918 (64)
LGN#1863 (77)
E
SA/PA angle distribution (% of cells in metaphase)
0
20
40
60
80
n.s.
wildtype (106)
control (79)
par1b-KD (68)
rescue (61)

Figure 6. LGN accumulates at the apicolateral subdomain in a Par1b-dependent manner and controls spindle orientation. **(A,B)** Localization of LGN (white outline arrowheads) in control (scrambled) and Par1b shRNA HepG2 cells. The apical domain (red arrowheads) is labeled with ABCC2 (A) or F-actin (B). Tight junctions are labeled with ZO-1 (B). The dashed line represents the SA. The solid line represents the PA. The outline diagrams ("PM domains") show the identity of the cell membranes of the dividing cells (#). Grey, red, orange, and green lines represent the basal, apical, lateral, and apicolateral plasma membrane domains, respectively. **(C)** Western blot analysis of LGN knockdown in HepG2 cells using two shRNA constructs. **(D)** Histogram analysis of SA/PA angles in LGN knockdown HepG2 cells indicating a loss of bias towards lower angles (0–30°) under LGN knockdown conditions. **(E)** Histogram analysis of wild type, control (scrambled) shRNA, Par1b knockdown, and Par1b rescued HepG2 cells shows a loss of apicolateral-directed spindle orientation under Par1b knockdown conditions. * $p < 0.05$. ** $p < 0.01$. n.s., not significant. Scale bars: 5 μm.

knockdown effectively abolished the bias in mitotic SA orientation towards smaller SA/PA angles (0–30°). An illustrative example of this is shown in Figures 6A, 6B, and S5D, and the quantifications are depicted in Figures 6E and S5C. The reintroduction of shRNA-resistant Par1b (Figure S5A) completely rescued this effect, and treatment of the cells with a scrambled shRNA without effect on Par1b expression did not affect the SA/PA distributions (Figure 6E, control and rescue). Similarly to in HepG2 cells, the knockdown of Par1b in WIF-B9 cells, or the expression of a dominant-negative Par1b mutant, caused a statistically significant shift in SA/PA angle bias from 0–30° to 31–60° and 61–90° (Figure S7A–S7C), underscoring that the role of Par1b mitotic spindle orientation is a feature of polarized hepatocytes and not only of HepG2 cells. Concomitant with the loss of bias towards 0–30° angles, live cell imaging showed that the frequency of cell divisions in which both daughter cells inherited part of the same apical lumen significantly increased upon Par1b depletion or expression of the Par1b-DN mutant (Figure S7D–S7F; Movie S9). Taken all together, our data suggest that, in cells with a hepatic type polarity, Par1b controls the apicolateral enrichment of LGN and, thereby, the apicolateral-directed orientation of the mitotic spindle to promote the asymmetric segregation of the apical plasma membrane domain to the two emerging daughter cells and to preserve the typical polarity phenotype of polarized hepatocytes.

DISCUSSION

This study demonstrates that mitotic hepatocytes asymmetrically segregate their apical plasma membrane domains to the emerging daughter cells during cell division. This is in striking contrast to the symmetric segregation of apical and basal surface domains observed in vitro and in vivo in simple epithelial cells such as those found in the neuroepithelium, kidney, and intestine (reviewed in [28]).

Our data indicate that this asymmetric inheritance of the apical plasma membrane domain in hepatocytes is dictated by an apicolateral-plasma-membrane-domain-directedorientation of the mitotic spindle. Interestingly, this apicolateral plasma membrane domain is a geometrically distinctive feature in cells with a (fetal) hepatic polarity phenotype (Figure 1A, apicolateral domain is green in the diagram). Indeed, the apicolateral domain represents the cell's contacting surface with the adjoining cell with which it shares an apical lumen, and can be distinguished from its contacting surface with other adjoining cells with which no apical lumens are (yet) shared (Figure 7, orange). This apicolateral subdomain has gone unnoticed, presumably because no functional relevance had been ascribed to it. Our data now demonstrate that Leu-Gly-Asn repeat-enriched protein (LGN) predominantly accumulates at this apicolateral domain during mitosis, both in rat liver hepatocytes in vivo and in polarized HepG2 cells in culture. Furthermore, NuMA-positive astral microtubules predominantly target this apicolateral domain in mitotic HepG2 cells. These observations are consistent with data from other cell systems in which LGN recruits NuMA on astral microtubules to the cell cortex (reviewed in [26,28,30]). Indeed, knockdown experiments demonstrate that LGN is necessary for orientating the SA predominantly towards the apicolateral domain. We propose that this apicolateral domain thus serves as an instructive positional landmark in hepatocytes for the polarized recruitment of LGN, which, in turn, is required for the predominantly apicolateral orientation of the mitotic spindle and asymmetric segregation of the apical domain to the nascent daughter cells.

In contrast to the apicolateral accumulation of LGN in dividing hepatocytes, LGN has been shown to accumulate at the apical plasma membrane domain in asymmetrically dividing Drosophila neuroblasts [47,149]. In epithelial cells, atypical protein kinase C at the apical plasma membrane has been proposed to exclude the apical recruitment of LGN [67,150]. Possibly, the presence of atypical protein kinase C at the apical bile canalicular plasma membrane domain in HepG2 cells and primary hepatocytes (unpublished data) may have similarly prevented the accumulation of LGN at the apical surface. But

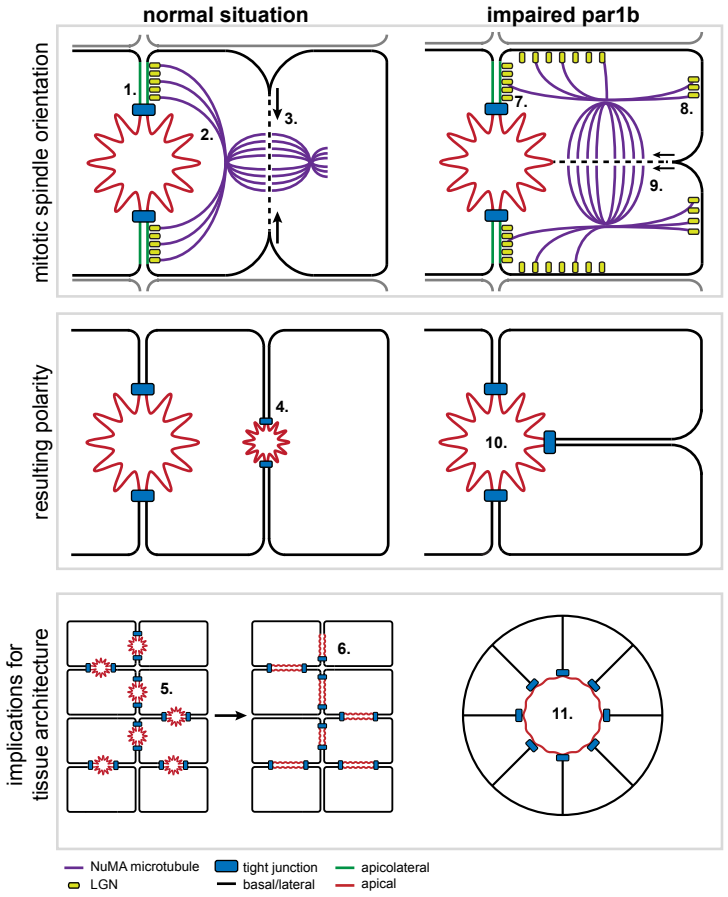

Figure 7. Implications of mitotic spindle orientation during the development of the unique liver architecture. **(1)** LGN localizes to the apicolateral plasma membrane area during hepatocyte cell division. **(2)** The mitotic spindle orients one of its (NuMA-containing) spindle poles towards the LGN-enriched apicolateral plasma membrane. **(3)** This orientation of the mitotic spindle results in the cleavage furrow not bisecting the apical plasma membrane, resulting in asymmetric segregation of the apical plasma membrane. **(4)** New apical surfaces are created de novo at the site of abscission. **(5)** During early liver development, apical pockets are created between hepatocytes. **(6)** These pockets grow out to bile canalicular/channel-like structures during later phases of liver development. **(7)** When Par1b is impaired, LGN migrates away from the apicolateral plasma membrane area and is subsequently found on basal or lateral membranes. **(8)** The mitotic spindle orients its poles towards LGN-enriched cortical areas. **(9)** The cleavage furrow has an increased chance of bisecting the apical plasma membrane, resulting in symmetric segregation of the apical plasma membrane. **(10)** Both cells now share the same apical surface ("simple" epithelial polarity). **(11)** Continued cell division likely results in the generation of "simple" epithelial cyst-like structures.

what caused LGN accumulation at the apicolateral subdomain and excluded it from the "common" lateral surface in hepatocytes? Our data implicate the polarity protein Par1b as a critical determinant for this. Indeed, upon knockdown of Par1b in HepG2 cells, LGN no longer accumulated predominantly at the apicolateral domain, but rather showed

additional localization at "common" lateral plasma membrane domains. In agreement with the occurrence of multiple sites of cortical LGN, NuMA-positive astral microtubules reached multiple sites at the cell surface. Concomitant with the altered distribution of LGN, knockdown of Par1b or expression of a nonfunctional Par1b mutant in HepG2 and WIF-B9 cells resulted in a loss of spindle orientation bias towards the apicolateral domain and promoted symmetric cell divisions that bisected the apical surface. Notably, in Par1b-depleted hepatic cells the loss of apicolaterally restricted LGN occurred while cells maintained their apicolateral domain. This suggests that Par1b translated the presence of the apicolateral domain as a positional landmark to cortical polarity—i.e., the apicolateral accumulation of LGN—in the mitotic cell. This is further supported by our observations that the overexpression of Par1b in simple epithelial MDCK cells coordinated the acquisition of a fetal hepatocyte polarity phenotype (and thus the establishment of an apicolateral domain) with apicolateral LGN recruitment during mitosis, spindle orientation, and asymmetric cell division. This demonstrates that the coordinated action of Par1b and LGN constitutes a fundamental part of the molecular mechanism that drives mitotic spindle orientation and asymmetric/symmetric apical plasma membrane inheritance. Further studies are needed to determine how Par1b precisely controls the exclusive apicolateral recruitment of LGN.

The orientation of cell division parallel to the apical–basal axis establishes cell fate specification, as has been shown in skin epithelial cells [42,43] and in neuroblasts [28], although not necessarily [63]. Apart from the asymmetric inheritance of apical plasma membrane proteins, we observed no overt signs of cell fate specification in dividing HepG2 cells. Live cell imaging showed that emerging daughter cells that did not inherit the apical plasma membrane domain were capable of establishing an apical plasma membrane domain and lumen with a new neighbor or the sister cell, and did not show distinct behavior when compared to the polarized daughter cell. We cannot, however, exclude the asymmetric acquisition of specific molecules that may have endowed one of the daughter cells with distinct capabilities. Although fetal liver development/patterning has not been experimentally tested, our findings may suggest that in early fetal hepatocytes the asymmetric segregation of apical domains during division may, in conjunction with a repolarization of non-polarized nascent daughter cells, promote the dissemination of isolated apical lumens throughout the proliferating cell mass. It can be speculated that, in vivo, such asymmetric-cell-division-driven propagation of biliary luminal pockets throughout the proliferative fetal liver parenchymal mass could facilitate the development of a branching canalicular network via concomitant or subsequent apical lumen expansion and fusion (Figure 7). Indeed, elongation

of bile canaliculi results from the expansion and fusions of numerous small isolated apical lumens [99,100,134] and as such would not necessarily require symmetric cell divisions. We propose that the Par1b-regulated spindle orientation via LGN and the resultant asymmetric inheritance of individual apical plasma membrane domains and lumens, as shown in this study, are made possible by and serve the unique polarized architecture of hepatocytes and, possibly, the liver parenchymal tissue.

To our knowledge, there have been no reports in the literature on human liver diseases associated with Par1b, LGN, or hepatic spindle disorientation. Defects in the orientation of the mitotic spindle apparatus may hamper the efficient development of bile canalicular networks during normal liver development or regeneration, or promote the development of cystic lumens, the latter process typically being driven by symmetric divisions [66,69,72]. In future studies Par1b knockout mice may be useful to investigate the role of Par1b in the formation of the bile canalicular network during embryonic liver development or regeneration after hepatectomy.

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AUTHOR CONTRIBUTIONS

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: CLS FL MB AB AM SVIJ. Performed the experiments: CLS FL MB MJMT. Analyzed the data: CLS FL QD AM SVIJ. Contributed reagents/materials/analysis tools: MJMT AB QD FL AM. Wrote the paper: CLS SVIJ.

SUPPLEMENTARY FIGURES

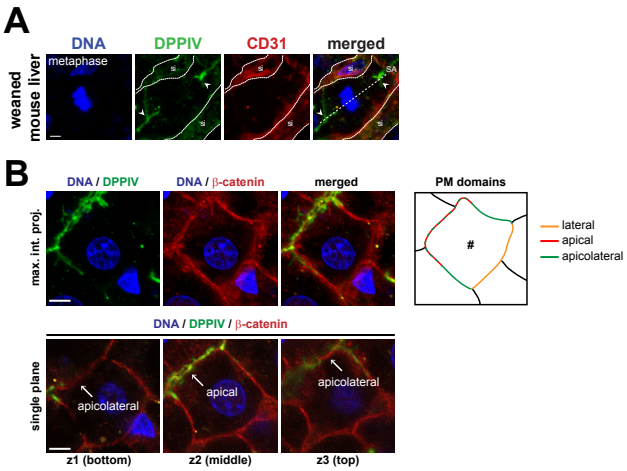

Figure S1. Mouse and rat hepatocytes orient their mitotic spindle axis towards the apicolateral subdomain. (A) Shown is a near-native tissue slice (100 µm) of weaned mouse liver stained for DNA, bile canaliculus (DPPIV/CD26), and the sinusoid (mouse, CD31). The SA intersects the apicolateral domain. Dotted white lines outline the sinusoid (si). **(B)** Immunofluorescence labeling of the bile canalicular protein DPPIV (green) and the cell–cell adhesion junction–associated protein beta-catenin in 2-d postnatal rat hepatocytes. Apicolateral and "common" lateral plasma membrane domains, color-coded in the diagram, can be distinguished. The # marks the cell for which the membranes were distinguished. See also Movie S1. Scale bars: 5 µm.

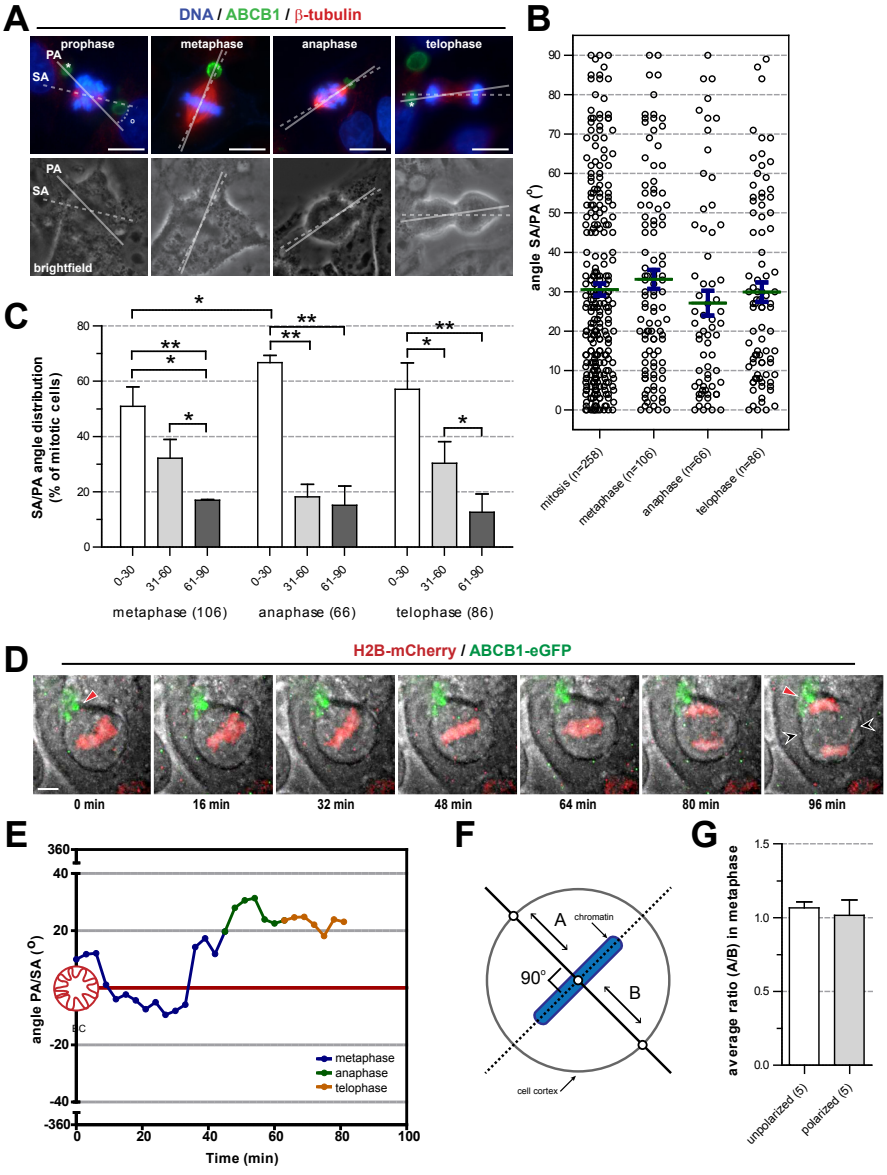
A
DNA / ABCB1 / β-tubulin
prophase
metaphase
anaphase
telophase
PA
SA
brightfield
B
angle SA/PA (°)
mitosis (n=258)
metaphase (n=106)
anaphase (n=66)
telophase (n=86)
C
SA/PA angle distribution (% of mitotic cells)
0-30
31-60
61-90
metaphase (106)
anaphase (66)
telophase (86)
D
H2B-mCherry / ABCB1-eGFP
0 min
16 min
32 min
48 min
64 min
80 min
96 min
E
angle PA/SA (°)
BC
metaphase
anaphase
telophase
Time (min)
F
A
B
chromatin
90°
cell cortex
G
average ratio (A/B) in metaphase
unpolarized (5)
polarized (5)

Figure S2. Hepatocytes predominantly orient their mitotic spindle axis towards the apicolateral subdomain and asymmetrically segregate their apical plasma membrane. **(A)** Illustration of HepG2 cells in various mitotic phases for which the SA/PA angle was calculated (the asterisk marks the apical domain to which the SA/PA angle was calculated). The apical domain is labeled with ABCB1. The microtubules of the mitotic spindle were labeled with β-tubulin. **(B)** Dot plot of SA/PA angles for dividing HepG2 cells for the various phases shown in (A). Shown is mean (green bar) and SEM (blue error bars). **(C)** Histogram analysis reveals a strong bias for HepG2 cells to divide with an SA/PA angle between 0° and 30° during metaphase, anaphase, and telophase. **(D,E)** A closer examination of the real-time dynamics of spindle orientation during mitosis by live cell imaging (D) (stills from Movie S2; DNA labeled by H2B-mCherry, the apical domain labeled by ABCB1-eGFP and red arrowheads; black arrowheads mark the ingressing cleavage furrow) reveals that the SA/PA angle oscillates between −15° and 15° relative to the apical–basal axis (E) (blue line; cell from Movie S2), while maintaining the same spindle pole facing the apicolateral domain. Prior to the onset of anaphase, the SA appears stabilized at a fixed orientation and shows minimal if any rotation during the subsequent course of mitosis (E) (green and orange lines; Movie S2). **(F,G)** The position of the mitotic spindle was calculated by taking the ratio of line A over B (F), indicating that the mitotic spindle is positioned in the middle of the dividing cell (G). * $p < 0.05$. ** $p < 0.01$. Scale bars: 10 µm (A) and 5 µm (D).

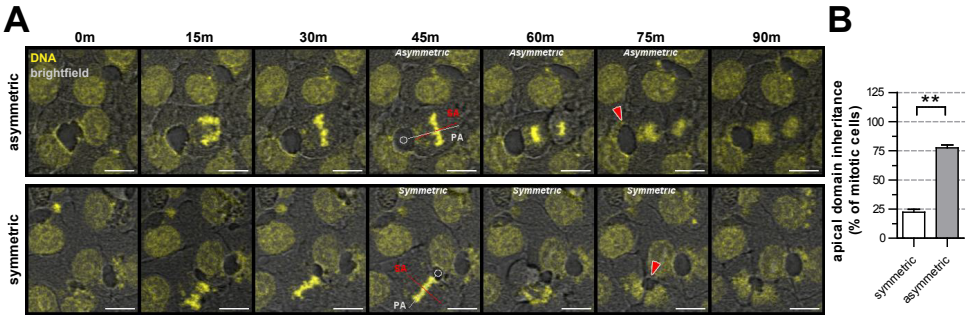

Figure S3. WIF-B9 cells segregate the apical plasma membrane and lumen asymmetrically during mitosis. **(A)** Stills from Movie S4. WIF-B9 cells, labeled with DRAQ5 to label chromatin/DNA, showing asymmetric and symmetric segregation of the apical plasma membrane (red arrowheads). **(B)** The graph represents a quantification of the asymmetry of apical domain inheritance in dividing WIF-B9 cells (live imaging; n = 27). ** $p < 0.01$. Scale bars: 10 µm.

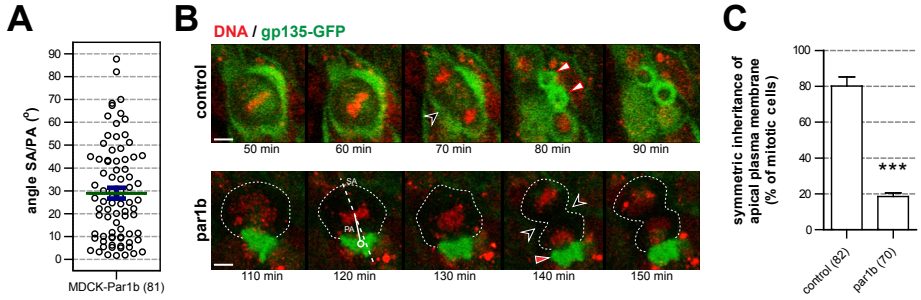

Figure S4. Asymmetric segregation of the apical plasma membrane domain in Par1b-overexpressing MDCK cells. **(A)** Dot plot of SA/PA angles for dividing MDCK-Par1b cells. Shown is mean (green bar) and SEM (blue error bars). **(B,C)** Time-lapse analysis (stills from Movie S6) (B) and quantification (C) of control and MDCK-Par1b cells, indicating symmetric and asymmetric inheritance of apical plasma membrane domains in control and MDCK-Par1b cells, respectively. *** $p < 0.001$. Scale bars: 5 µm.

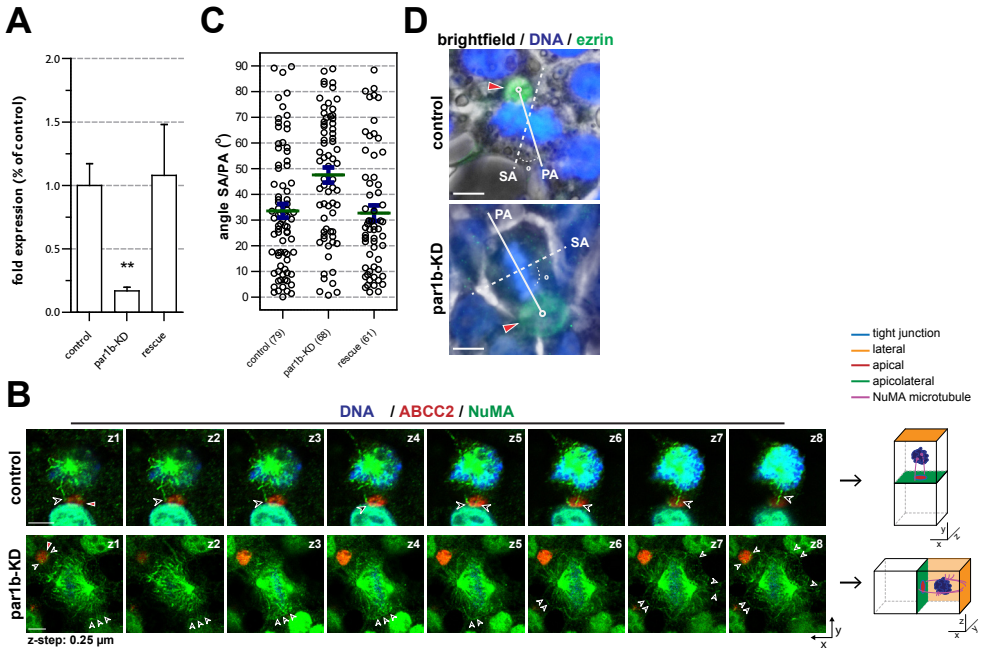

Figure S5. Par1b regulates apicolateral-directed spindle orientation in HepG2 cells. **(A)** Quantification of Par1b knockdown in HepG2 cells by quantitative PCR. **(B)** Stills from Movies S7 and S8, showing cortical NuMA (black arrowheads) at the apical domain and at both the apical and lateral membranes in control and Par1b knockdown HepG2 cells, respectively. **(C)** SA/PA angle was calculated for control (scrambled) shRNA and Par1b knockdown HepG2 cells (fixed) as indicated in **(D)** and plotted as depicted. All figures: red arrowheads mark the apical domain. The outlines show the identity of the cell membranes of the dividing cells (#) shown in (A). Red, orange, and green lines represent the apical, lateral, and apicolateral plasma membrane domains, respectively. ** $p < 0.01$. Scale bars: 5 µm.

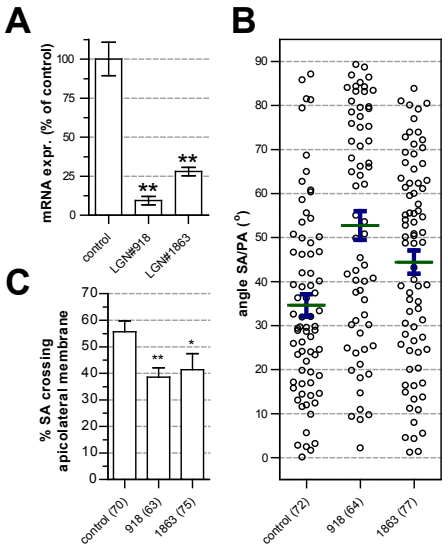

Figure S6. Short hairpin RNA targeted against LGN results in the depletion of the LGN protein in HepG2 cells, which, in turn, perturbs the apicolateral orientation of the mitotic spindle apparatus. **(A)** Real-time PCR analysis of the knockdown efficiency of the two LGN constructs used in this study. **(B)** Dot plot of SA/PA angles of HepG2 cells in metaphase under control and LGN knockdown conditions. Shown is mean with SEM. **(C)** Shown is the percentage of HepG2 cells with SA crossing the apicolateral membrane under control and LGN knockdown conditions, indicating reduced apicolateral spindle orientation under LGN knockdown conditions. * $p < 0.05$. ** $p < 0.01$.

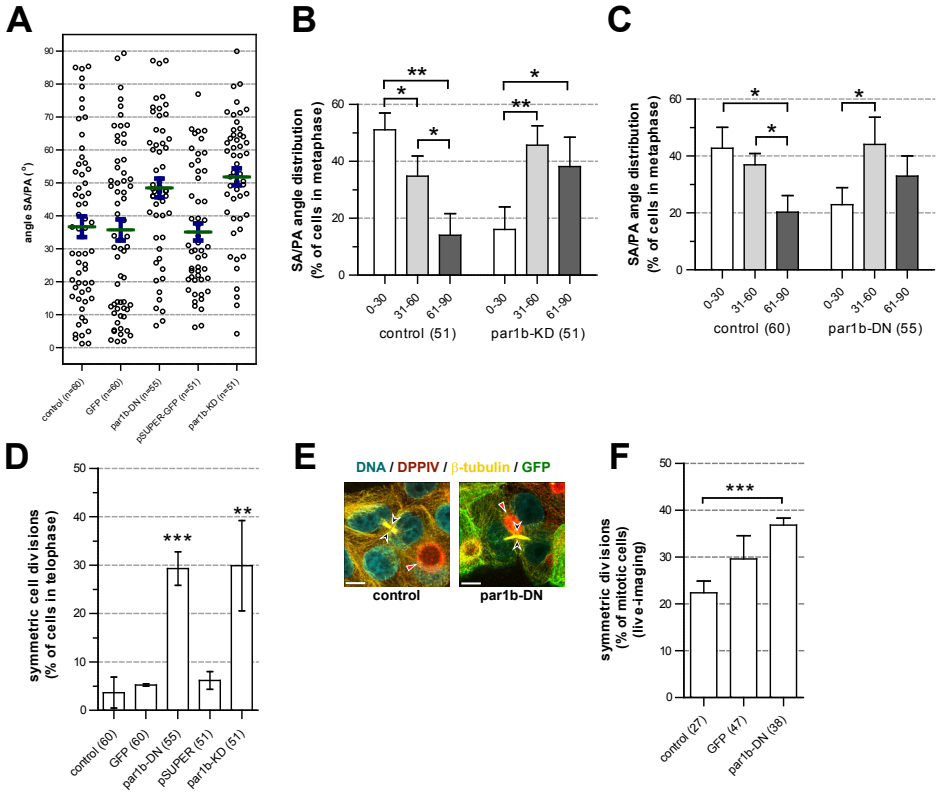

Figure S7. Knockdown of Par1b in WIF-B9 cells alters the orientation of the mitotic spindle apparatus relative to the apical polarity axis. **(A)** Dot blot showing individual SA/PA angles for dividing WIF-B9 cells under the depicted conditions. Shown is mean with SEM. **(B)** Histogram analysis of control (pSUPER) and Par1b-KD WIF-B9 cells indicating reduced apicolateral-subdomain-oriented spindle orientation (reduced bias towards lower angles [0–30°]) during Par1b depletion. **(C)** Histogram analysis of control (GFP) and Par1b-DN-GFP-expressing WIF-B9 cells indicating reduced apicolateral-subdomain-oriented spindle orientation (reduced bias towards lower angles [0–30°]) when Par1b function is perturbed. **(D)** WIF-B9 cells expressing Par1b-DN and Par1b knockdown cells in metaphase were scored for symmetric or asymmetric segregation of the apical plasma membrane. Reduced Par1b activity increased symmetric inheritance of the apical plasma membrane. **(E)** Illustrations of dividing control and Par1b-DN-expressing WIF-B9 cells. DPPIV marks the apical domain, β-tubulin marks the microtubules of the mitotic spindle. **(F)** Symmetry of cell division was also quantified during live imaging of WIF-B9 cells. Black arrowheads mark the ingressing cleavage furrow (midbody, site of cytokinesis). * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. Scale bars: 5 µm.

SUPPLEMENTARY MOVIES

Supplementary movies and legends are available online:
http://dissertations.ub.rug.nl/faculties/medicine/2014/c.l.slim/

SUPPLEMENTARY TABLES

Supplementary Table 1. Commercial antibodies used in this study.

Target	Species	Company	Catalog#/Clone
aPKC	rabbit	Santa Cruz Biotechnology	sc-216
α-tubulin	rat	Abcam	Ab6160
ezrin	rabbit	Santa Cruz Biotechnology	sc-20773
abcc2	mouse monoclonal	Enzo Life Sciences	ALX-801-016
NuMA	rabbit	Abcam	ab36999
ß-tubulin	mouse monoclonal	Millipore	MAB3408
ZO-1	mouse monoclonal	Life Technologies	clone 1A12
DPPIV	mouse monoclonal	AbD Serotec	MCA924R
DPPIV	goat polyclonal	R&D Systems	AF954

Supplementary Table 2. RNA interference target sequences for HepG2 cells. Listed are the sense sequences used to generate oligos according to the pLKO manual.

Target	Sequence
Par1b	CAGCAAGAGAGGCACTTTAGA
LGN#918	AAACGAATTCCTCCACTAAAG
LGN#1863	AAACGAATTCCTCCACTAAAG

Supplementary Table 3. Real-time PCR primers used in this study. Listed are forward and reverse primer DNA sequences.

Target	Forward	Reverse
Par1b	GCAGCCCCACATTGGAAAC	CACAGCTACCTCTTTCCCAGTCA
HMBS	GGGAAACCTCAACACCCGGCT	ATCCTGGTTGTGCCAGCCCAT
GAPDH	CATTTCCTGGTATGACAACG	GTCCAGGGGTCTTACTCCTT
LGN	ATTAGTGACTGCTTTGGGTG	AGTGTCTTCTTGTAGTATTCCGAG

CHAPTER 5

The special case of hepatocytes: unique tissue architecture calls for a distinct mode of cell division

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ABSTRACT

Columnar epithelia (e.g., kidney, intestine) and hepatocytes embody the two major organizational phenotypes of non-stratified epithelial cells. Columnar epithelia establish their apical and basal domains at opposing poles and organize in monolayered cysts and tubules, in which their apical surfaces form a single continuous lumen whereas hepatocytes establish their apical domains in the midst of their basolateral domains and organize a highly branched capillary luminal network, the bile canaliculi, in which a single hepatocyte can engage in lumen formation with multiple neighbors. To maintain their distinct tissue architectures, columnar epithelial cells bisect their luminal domains during symmetric cell divisions, while the cleavage furrow in dividing hepatocytes avoids bisecting the bile canalicular domains. We discuss recently discovered molecular mechanisms that underlie the different cell division phenotypes in columnar and hepatocytic model cell lines. The serine/threonine kinase Par1b determines both the epithelial lumen polarity and cell division phenotype via cell adhesion signaling that converges on the small GTPase RhoA.

SHORT COMMUNICATION

Epithelial cells constitute the biggest cell pool in the mammalian organism, with ~60% of mammalian cell types being of epithelial or epithelial-derived origin [1]. Epithelial cells are found in a wide variety of tissues, such as the skin, lung, intestine, kidney, and liver, where they are situated at the interface between the organisms exterior and interior environment. Their major function is to protect the organism's interior milieu (i.e., the blood) by physically separating it from the exterior environment, and also to regulate the transport of molecules (e.g., nutrients) between the environments [1,2]. Epithelial cells are polarized cells in the sense that they have specific plasma membrane domains (also referred to as surfaces) that face either the exterior or the interior environment, or neighboring cells. The apical membrane faces the external environment or 'lumen' of the organism, such as the interior of the gut or lung. The basal membrane faces the interior milieu of the organism and is typically in contact with the extracellular matrix (ECM) and, ultimately, underlying blood vessels. The lateral plasma membrane domains contact neighboring cells via cell-adhesion protein complexes such as adherens junctions, desmosomes, and gap junctions [3]. The basal and lateral membrane domains are often commonly referred to as the basolateral membrane, and the apical and basolateral plasma membrane domains are separated, and its protein and lipid composition maintained, by tight junctions [3–5]. Most epithelial organs are created by epithelial cells of the columnar (they are typically taller than they are wide) polarity type and are aligned in tight single-cell monolayers that wrap around a central cavity or lumen, a hallmark of columnar polarity [1]. Architecturally, columnar epithelial cells create hollow tubes that ultimately develop into interconnected tubular networks (Fig. 1, "Columnar").

However, not all epithelial tissues develop columnar type of epithelial polarity and tubular architecture. The liver is an important metabolic organ and is responsible for the generation of bile salts, cholesterol homeostasis, plasma protein production, detoxification of the blood, and hormone and cytokine production. The epithelial cell of the liver, the hepatocyte, constitutes ~78–85% of the liver cell mass [87,88] and provides most liver functions. In the adult healthy liver, hepatocytes are aligned in one or two-cell thick cords and are highly polarized. Similar to columnar epithelial cells, the basal membranes of hepatocytes are in contact with the ECM and blood via endothelial-lined sinusoids (also known as the space of Disse), and their lateral membranes are used to contact neighboring hepatocytes. During liver development, hepatocytes form small apical domains enclosed by tight junctions at the lateral membrane of two hepatocytes,

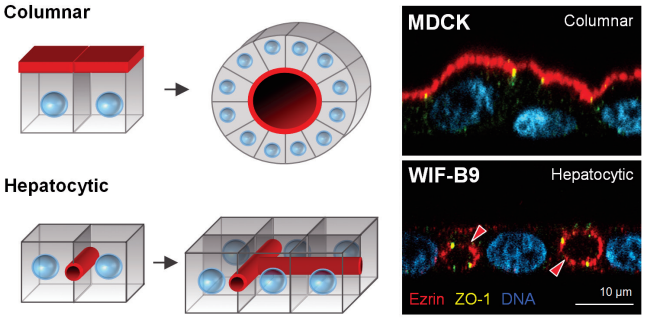

Figure 1. Columnar vs. hepatocytic polarity. Columnar epithelial cells form monolayers where multiple cells surround a central lumen (i.e., columnar polarity), whereas hepatocytes organize around tubular networks were the luminal domain is shared by no more than two cells (i.e., hepatocytic polarity), and each cell can have multiple luminal domains. Red arrowheads indicate the luminal domains marked by Ezrin. MDCK and WIF-B9 cells are kidney- and hepatocyte-derived culture models, respectively.

that can later in development merge together and form canalicular structures that circumvent entire hepatocytes, a hallmark of hepatocyte polarity [132–134,147,202]. Hepatocytes use the canaliculi to secrete and drain bile and it is commonly referred to as the bile canalicular network (Figure 1, "Hepatocytic").

It is important to realize that while adult hepatocytes show polarized plasma membrane domains like other epithelial cells, their polarity phenotype and the 3-dimensional tubular architecture that they create is different from a columnar epithelium. As described above, columnar epithelial cells form monolayers of multiple cells surrounding a central lumen (i.e., columnar polarity), but hepatocytes do not. In fact, from the apical domain point-of-view, the apical domain of hepatocytes is only shared by not more than two hepatocytes (i.e., hepatocytic polarity). It is because of this specific apical domain organization that bile canaliculi are able to completely circumvent entire hepatocytes.

How tubular networks are formed is a subject of intense research. Recently, the orientation of cell division was found to be an important design principle for generating and maintaining columnar epithelial tissue (i.e., tubular) architecture, and failure to properly orient cell divisions correlates with tumorigenesis [1,56–60]. The orientation of the mitotic spindle during mitosis dictates the position of the cleavage furrow, which is established perpendicular to the spindle pole axis. Thereby, mitotic spindle orientation guides both the positioning of the daughter cells within the epithelial tissue and the equal or asymmetric inheritance of cellular domains and cytoplasm by the two daughters. Columnar epithelial cells arranged in monolayers predominantly align their mitotic spindle with the substratum (e.g., ECM) and divide symmetrically, while

preserving their membrane domain identities, as shown for kidney-derived MDCK cells [61]. That is to say, epithelial cells symmetrically 'segregate' their apical and basolateral membrane domains to both daughter cells during cell division, and the daughter cells are then positioned in the plane of the monolayer, preserving tubular architecture (Figure 2A "Columnar" and Figure 2B, "Control").

However, symmetric inheritance of plasma membrane domains poses a problem for hepatocytes and hepatic polarity development, as this would induce and enforce the generation of columnar tubular structures with columnar polarity, and would therefore be incompatible with the formation of a canalicular network that is so unique for the liver architecture. Thus, hepatocytes must use a different orientation of the cell division mechanism to maintain hepatocyte polarity, and ultimately allow for the formation of the bile canalicular network. Indeed, earlier work in fixed rat liver tissue slices revealed that after partial hepatectomy proliferating hepatocytes asymmetrically segregate apical plasma membrane domains during cell division [142,143], though the 3-dimensional mechanics and molecular mechanisms have, until now, remained unknown. Using HepG2 and WIFB9 cell lines as in vitro cell models for hepatocyte polarity [10,97,102] and studying cell division in regenerating rodent livers after partial hepatectomy, we found that, in compliance with earlier work, hepatocytes display a different mode of cell division orientation compared with columnar epithelial cells [86,158] (cf. Chapter 2). Specifically, hepatocytes orient their mitotic spindle poles toward an area near the apical plasma membrane domain, which we call the apicolateral membrane domain, and during cytokinesis asymmetrically segregate their apical plasma membrane domain to daughter cells (Figure 2A, "Hepatocytic"). By doing this, hepatocytes maintain their hepatic polarized state, and would ultimately be allowed to form and maintain the bile canalicular network to serve the unique liver architecture.

To understand mechanistically how the two epithelial cell division phenotypes are linked to the two distinct polarity phenotypes, it helps to break down mitotic spindle orientation into spindle position in the x-y plane (birds-eye or planar view) and in the x-z plane (side view). In fact, most studies consider only one of these dimensions. In the x-z view, columnar, symmetrically dividing cells align their metaphase spindle with the basement membrane. This horizontal spindle orientation depends on the local positioning of two cortical cues that capture the two sets of astral microtubules at opposite lateral cell membranes in equal-distance from the basal domain. The attachment cues are an evolutionary conserved module that consists of the $G\alpha_i$ subunit of a trimeric G-protein that is anchored to the cortex via a myristoyl group and its binding partners,

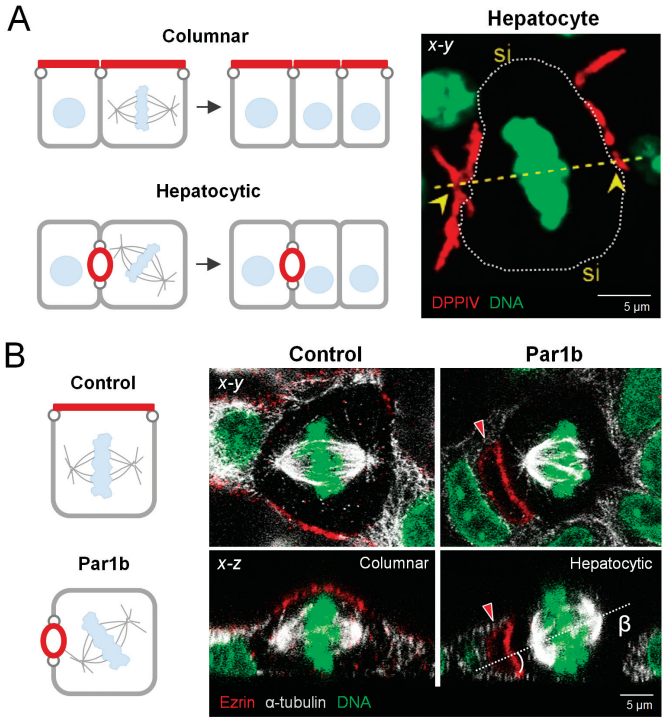

Figure 2. Columnar and hepatocytic division phenotypes are regulated by Par1b. **(A)** Columnar epithelial cells orient their metaphase plates perpendicular to the lumen. The resulting cleavage furrow bisects their luminal surface (red domains in the schematics, marked by dipeptidyl peptidase-IV (DPPIV)). Hepatocytes attach their astral microtubules adjacent to their luminal domain(s), thereby avoiding the bisection of their lumina during cell divisions. In cultured hepatocytic cells with a single luminal surface, as depicted in the schematic, the luminal domain is distributed to only one of the daughters. In multipolar hepatocytes in vivo, as shown in the fluorescent image on the right, one of the two DPPIV-positive luminal surfaces (yellow arrowheads) will segregate to each daughter. Si = sinusoids. **(B)** Par1b overexpression in MDCK cells promotes polarization with lateral rather than apical lumina (see x-z views, the apical domains (red) are marked by Ezrin) and mitotic spindles that are oriented toward the lateral lumen, instead of aligning with the basal surface. The β angle represents the angle between the spindle axis (dashed line) and the substratum.

the proteins LGN (leucine-glycine-asparagine repeat protein) and NuMA (nuclear and mitotic apparatus) (reviewed in ref. [39]). The latter is a nuclear protein that only becomes available for cortical complex formation after the nuclear envelope breaks down at the onset of prophase. NuMA mediates the interaction of astral MTs with the cortex via Dynein, a minus-end directed microtubules (MTs) motor. When anchored to the membrane by NuMA and walking along astral MTs toward the spindle poles, Dynein can exert pulling forces on the MTs that bring the spindle into place. When either Gαi, LGN, NuMA, or Dynein are depleted, spindles no longer align with the basal surface but become "tilted." A similar phenotype is also observed when the spindle attachment module is present along the entire cell cortex. In HeLa cells, the restriction of NuMA to

a tight patch on the lateral domain is due to β1-integrin mediated signaling processes that involve PI3K and Abl kinase activity as well as myosin X [33,203]. Curiously, none of these ECM-signaling events appeared to operate in polarized MDCK cells when they were tested side-by-side with HeLa cells, which lack cell-cell adhesion junctions. Instead, independent work in Drosophila neuroepithelia and in MDCK cells established cell-cell adhesion proteins as the domineering cues in polarized epithelial cells, specifically the Adenomatosis Polyposis coli protein (APC), which has MT-tip binding abilities and could therefore directly capture astral MTs [204]. In MDCK cells, APC-depletion or depletion of E-cadherin, which is instrumental in recruiting APC to adherens junctions (AJ), did not prevent cortical LGN/NuMA, but nevertheless caused tilted spindles [205]. These findings led to the concept that ECM-signaling governs x-z spindle position via LGN/NuMA in non-adherent cells while cell-cell adhesion proteins serve as spindle attachment cues in polarized epithelia. Several findings, however, didn't fit this simple model: β1-integrin depletion in Drosophila follicle epithelia caused tilted spindles and integrin signaling determined spindle positioning in mammalian basal keratinocytes; thus ECM-signaling does have a dominant role in epithelial spindle orientation in vivo [41,206]. Furthermore, the LGN/NuMA module, which in mitosis colocalizes with adhesion markers at the lateral domain, overrides any cell-cell adhesion-mediated cues when it is ectopically activated in MDCK cells [66]. We have now demonstrated that function-blocking β1-integrin antibodies indeed abolish spindle alignment with the substratum in MDCK cells, and further determined that the recruitment of LGN/NuMA to the metaphase cortex is dependent on collagen-IV mediated ECM-signaling in MDCK and HepG2 cells [158], although laminin-1 might also play a role (cf. Chapter 3). In both cell lines, the position of a NuMA patch always correlated with a spindle pole facing NuMA. How does ECM/integrin signaling at the basal domain translate into discrete LGN/NuMA recruitment at the lateral cell cortex in epithelial cells? When cells enter mitosis they disassemble their focal adhesions leading to cell rounding and their cell cortex becomes stiff. Both these changes, one at the basal, the other at the lateral surface, are known to require RhoA activity [207]. These observations made us wonder whether RhoA signaling could link basal ECM-signaling to lateral membrane organization. Indeed, we found, utilizing a FRET-based biosensor, that the presence of NuMA at the cortex always coincided with high RhoA activity, while RhoA was less active at the NuMA-negative cortex. Furthermore, depletion of RhoA or pharmacological inhibition of the RhoA effector Rho-kinase abolished LGN and NuMA from the metaphase cortex and resulted in tilted spindles, and HepG2 cell multilayering [139]. Thus, ECM-signaling appears to drive NuMA positioning by activating RhoA at discrete cortical sites. What are those sites? In MDCK and HepG2 cells NuMA localizes where cell-cell adhesion

junctions are present. They are connected to a circumferential actin belt that is under tension and likely requires RhoA to sustain high myosin II activity. Although we have not tested this hypothesis directly, we observed that non-polarized mitotic HepG2 cells lacked patches of high RhoA activity and were deficient in the recruitment of NuMA. Therefore, adherens junctions are good candidates to serve as sites of high RhoA activity required for LGN/NuMA recruitment and might function synergistically with the ECM signals to position the spindle parallel to the substratum in MDCK cells.

Spindle orientation in the x-y dimension also depends on ECM-signaling mechanisms [177]. When mitotic cells round up, their sole connections to the substratum are thin retraction fibers that correspond to the former cell adhesion points. The position of these retraction fibers serves as guideposts for the placement of the spindle. It is the tension in these fibers, which pin the cell down like the guylines of a tent, that convey a signal for x-y spindle positioning. It is tempting to speculate that RhoA activity is highest where retraction fibers are most abundant and attracts the $G\alpha_i$/LGN/NuMA module to these x-y positions (Figure 3, HeLa). Polarized epithelial cells have few focal adhesions and consequently feature few retraction fibers in mitosis. It is conceivable, as discussed above, that the cell-cell adhesion belt provides the RhoA cue in this case. However, the adhesion belt in monopolar columnar epithelial cells spans the entire cell circumference, suggesting that x-y spindle orientation in columnar epithelial cells is either random or that symmetry is broken by upstream signals that are likely non-cell autonomous. The latter would dictate the direction into which an epithelium expands. For columnar polarized epithelial cells growing as monolayers in culture, x-y spindle orientation is indeed unimportant. That LGN/NuMA are nevertheless restricted to two crescents rather than forming a continuous belt (Figure 3, MDCK), can be explained by removal of LGN/NuMA from the areas of the x-y cortex that are not initially involved in MT-anchoring: chromatin that aligns at the metaphase plate emits a gradient of active Ran GTPase that antagonizes cortical LGN/NuMA at sites where it comes closest to the cortex, which is perpendicular to the spindle poles and the anchored astral MTs [208]. In contrast to columnar epithelial cells, the lumen architecture of HepG2 cells results in a sub-luminal LGN/NuMA belt that is too narrow to anchor both astral MT fans. Even if the spindle would manage to curl around the luminal domain, the supposed Chromatin-Ran-gradient would likely remove the entire LGN/NuMA population. Instead, the subluminal NuMA patch anchors only one astral MT fan with the other facing the opposite basolateral surface. LGN/NuMA thereby serves as cue for the x-y spindle orientation. Indeed, depletion of LGN abolished the alignment of the spindle with the luminal domain in HepG2 cells [86] (cf. Chapter 2). In multipolar hepatocytes, a second

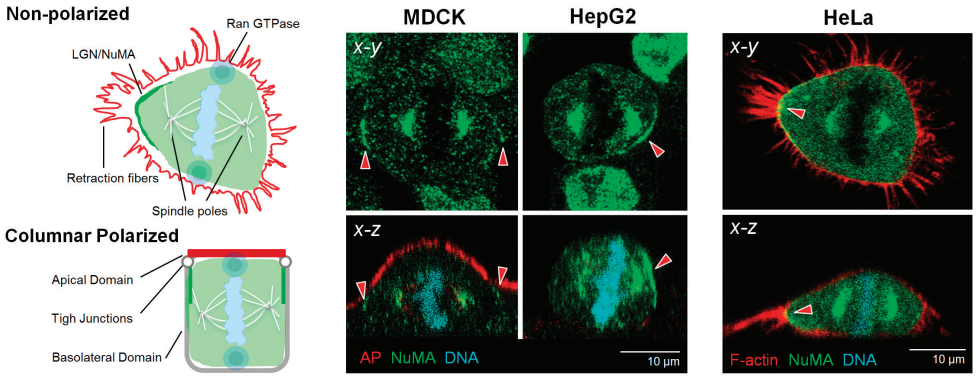

Figure 3. NuMA localization in polarized MDCK and HepG2 and in non-polarized HeLa cells. In polarized epithelial cells, such as MDCK and HepG2 cells, cortical NuMA in metaphase localizes below or adjacent to the luminal domain, coinciding with cell-cell adhesion sites (white arrowheads). The schematic illustrates the example of columnar epithelia ("Columnar Polarized"), In transformed epithelial cells such as HeLa cells, which lack adherens junctions, cortical NuMA coincides with the strongest retraction fibers (schematic "Non-Polarized"). In both instances, these are cortical areas under tension that likely feature high RhoA activity. Metaphase chromatin emits a Ran GTPase gradient that antagonizes cortical NuMA where the chromosomes come closest to the cortex, (blue circles in the schematics), resulting in two NuMA crescents. F-actin and an apical marker (aminopeptidase, AP) are in red.

sub-luminal domain provides the anchor site for the other set of astral MTs (see Figure 2A). Thus, in MDCK cells, where spindle orientation in the x-y position is less important than in the x-z position for the maintenance of cell polarity and tissue architecture, Gαi/LGN/NuMA ensure spindle alignment with the basal domain but do not define its x-y-orientation. In HepG2 cells where x-y spindle orientation is equally important, the NuMA module also ensures that one of the spindle poles faces the luminal region in the x-y dimension.

Intuition suggests that the many molecular aspects that amount to the distinct polarity phenotype and tissue organization of monolayered epithelial cells and hepatocytes must result from many molecular signaling pathways and processes that are fundamentally distinct in these epithelia. It is remarkable therefore, that a ubiquitously expressed serine/threonine kinase, Par1b/MARK2 can single-handedly convert the two phenotypes into each other in vitro. Ever since one of our labs reported that Par1b overexpression in MDCK cells causes a switch to a hepatocyte lumen polarity phenotype as found in cultured hepatocytic HepG2 and WIFB cell lines [120,122], we wondered how far the phenotypic conversion goes. We have now demonstrated that MDCK-Par1b cells, like the hepatocytic lines, also feature tilted metaphase spindles that orient toward their lateral luminal domain and give rise to asymmetric divisions and asymmetrical inheritance of apical plasma membrane domains (cf. Chapter 2) [86,158] (Figure 2B).

Remarkably, Par1b not only promotes both these aspects of hepatocyte polarity in MDCK cells, but does so via common signaling mechanisms: Par1b inhibits the deposition of a basement membrane [209], and both Par1b-phenotypes are overcome when the basal lamina deposited by MDCK-Par1b cells is supplemented with exogenous collagen-IV [158]. Defective ECM-signaling in turn reduces RhoA activity. Inhibition of RhoA indeed causes tilted spindles and lateral lumen polarity in MDCK cells suggesting that the two polarity aspects are intimately linked. Our recent evidence suggests that the converse also applies, namely that HepG2 cells adopt features of the columnar phenotype when Par1b levels are reduced. Par1b-depletion in columnar epithelia leads to a disorganized monolayer, and complete abrogation of all Par1 activity (there is at least one additional Par1 isoform present in most cells) likely interferes with cell-cell adhesion [210]. Reduced Par1b levels in WIF-B9 cells resulted in areas of the monolayer that exhibited a chickenwire arrangement of tight junction and apical junctional markers and in the establishment of a luminal domain at the apex, although the phenotype reversal was not perfect and many cells simply lost polarity. The metaphase spindle in Par1b-depleted HepG2 cells always aligned with the substratum as observed in columnar epithelial cells, which resulted in symmetric divisions where the apical domain was divided between daughters. Even in HepG2 cells that maintained lateral lumen organization, the x-y spindle alignment mechanism was abolished, resulting in more divisions in which the luminal domain was divided between daughters. Altogether, these features resemble the domain and spindle organization of MDCK cells. The combined gain and loss-of function data thus implicate Par1b as a key determinant in the branching of the two epithelial phenotypes that are exemplified by kidney and hepatocyte epithelia.

CHAPTER 6

Summary and perspectives

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SUMMARY

The liver contains two epithelial cells types that each exhibit a fundamentally distinct type of cell polarity, and develop distinct tubular networks. Cholangiocytes, which are non-stratified (i.e., simple) epithelial cells, are apical-basal polarized and are aligned in monolayers surrounding a single central lumen. By doing so, they form the larger bile-collecting tubes of the biliary tree. Hepatocytes show a distinct type of polarity, hepatocyte polarity, with apical domains and lumens at the lateral membranes instead of at the cell apex, and form a bile canalicular network that drains bile from the hepatocytes towards the bile ducts. While the lumenogenic principles of simple epithelial cells and cholangiocytes are becoming clear, how hepatocytes regulate their unique type of cell polarity and consequently form the bile canalicular network is not understood. Therefore, the main objective of this thesis was to investigate the lumenogenic mechanisms that hepatocytes use to regulate and maintain their polarized architecture, and how these mechanisms may be translated into the formation of the bile canalicular network. Simultaneously, this thesis attempted to elucidate the principles that govern the formation of two types of epithelial cell polarity and tubular systems in the liver.

From earlier studies it has become clear that the orientation of cell division, which determines the positioning of daughter cells and inheritance of plasma membrane domains, is an important lumenogenic principle in simple epithelial lumenogenesis. Simple epithelial cells (including cholangiocytes) that are aligned in monolayers typically align their mitotic spindle with the substratum or extracellular matrix (that is, perpendicular to the apical-basal polarity axis) and, consequently, symmetrically segregate their plasma membrane domains to both daughter cells (that remain positioned in the monolayer). Hereby, simple epithelial cells preserve their polarized architecture and tubular integrity. In developing hepatocytes, however, this type of cell division is incompatible with the maintenance of hepatocyte polarity, and would induce the formation of simple epithelial polarity and larger lumens or tubules surrounded by multiple cells, rather than an anastomosing canalicular network. In Chapter 2 we show for the first time that hepatocytes display a unique and hepatocyte-specific mode of cell division and asymmetrically segregate their apical plasma membrane(s) during cell division. This mode of cell division is controlled by the hepatocyte-specific apicolateral localization of mitotic spindle-orienting cortical cues LGN and NuMA. The apicolateral location of these cues are in turn regulated by the polarity protein Par1b. When overexpressed in simple epithelial MDCK cells, Par1b induces the formation of hepatocyte polarity in simple epithelial cells. Conversely, knockdown of Par1b in HepG2 and WIFB9

cells caused a redistribution of LGN and NuMA to other plasma membrane domains, resulting in the removal of the apicolateral spindle pole orientation bias and the generation of cells with a single central lumen shared by multiple cells, a hallmark of simple epithelial polarity. Notably, mitotic spindle pole orientation in HepG2 cells is dependent on the apicolateral localization of LGN, as knockdown of LGN in HepG2 cells abolished the apicolateral spindle pole orientation bias.

These data suggest a model where hepatocytes use a hepatocyte-specific mode of cell division, guided by the Par1b-dependent apicolateral localization of mitotic spindle-orienting cues, to asymmetrically segregate apical plasma membrane domains and lumens during development of hepatocyte polarity and the bile canalicular network. Hereby, hepatocytes preserve their polarized architecture, and ultimately allow the formation of a bile canalicular network, rather than simple epithelial tubes. This model is in line with earlier observations in the developing liver, where hepatocytes form small apical lumens between individual hepatocytes throughout the liver parenchyma that can ultimately merge to form the bile canalicular network. Interestingly, we found that new lumens could form de novo at the site of cytokinesis, and propose that hepatocytes may use a hepatocytic mode of cell division to disperse multiple apical lumens throughout the liver mass that can later develop into bile canaliculi. Interestingly, asymmetric cell division was until now mainly associated with cell fate specification or tissue stratification (e.g., in the skin). In this thesis, we have shown for the first time the existence of a cell type, the hepatocyte, that uses asymmetric cell division solely for the purpose of maintaining its polarized architecture. Here, unpolarized daughter cells remain hepatocyte-specified and can form new apical domains and lumens de novo, facilitating the formation of the hepatocyte mass and bile canalicular network.

The basement membrane, onto which simple epithelial cells such as cholangiocytes normally reside, is important for the generation of simple apical-basal epithelial cell polarity and tubes. Consequently, it is also involved in controlling the orientation of cell division. Hepatocytes are in contact with a distinct type of ECM that lacks a basement membrane configuration, and display hepatocyte polarity. Whether the microenvironment is an important determinant in development distinct types of cell polarity in the liver is not clear. In Chapter 3 we have studied the role of the microenvironment composition on the polarized architecture of hepatocytes. We found that exposure of HepG2 cells to a basement membrane extract (BME) induced cell clustering and a phenotypic switch from hepatocyte-to-cholangiocyte type polarity that includes formation of cystic (i.e., acinar) structures that resemble simple epithelial polarity.

The basement membrane component laminin-1 was sufficient for cell clustering and development of these cystic structures. The conversion of hepatocyte-to-cholangiocyte type polarity was dependent on the orientation of the mitotic spindle and controlled by mitotic spindle-orienting cortical cues Gαi and LGN (cf. Chapter 2), which were redistributed away from the apicolateral membrane upon BME exposure, perturbing apicolateral spindle pole orientation. This likely altered the orientation of cell division and induced the occurrence of symmetric inheritance of apical plasma membrane domains and formation of simple epithelial polarity and tissue structure. Interestingly, the phenotypic conversion of hepatocytes on BME did not coincide with significant changes in transcriptional profiles, indicating that polarity phenotypes in the liver can be uncoupled from gene-expression profiles.

The data described in this chapter suggest that the specific composition of the microenvironment (e.g., presence or absence of a basement membrane) in the developing liver is an important regulatory determinant of the type of cell polarity that is developed by hepatic epithelial cells. How distinct types of ECM are deposited in the liver is not entirely clear, although many different cell types are likely to be involved [125,160]. Interestingly, Par1b, which controls the positioning of mitotic-spindle orienting cues in hepatocytes, also controls ECM signaling and deposition is MDCK cells [158,209]. Thus, via controlling the location, levels, composition, and signaling of the ECM, Par1b may be an important intrinsic regulator of cell division and the development of distinct polarity phenotypes in the liver.

Phenotypic conversions as described in this chapter are known to occur during a liver injury-triggered process called ductular reaction. Importantly, ductular reaction is known to correlate with the deposition of basement membrane-like ECM within the hepatocyte microenvironment. Speculatively, duct-like configurations may be the result of mitotically-activated hepatocytes that, via altered cell division mechanics, have adopted cholangiocyte-like polarity and architecture. Thus, our data may provide a novel mechanism behind the formation of ductal structures in ductular reaction. Also, we propose that it is the combined effect of changes in the cellular microenvironment with the induction of proliferative activity that enables these phenotypic conversions.

During early stages in the development of simple epithelial polarity and tubes, cells form a single apical domain that via lumenogenic mechanisms such as symmetric cell division and polarized intracellular transport is allowed to grow out and develop into a single central lumen surrounded by a sheet of epithelial cells. That is to say, simple

epithelial cells commit fully to the formation of a single apical domain and lumen during lumenogenesis. Hepatocytes show a distinct type of cell polarity and generate a distinct type of tubular system. Whether hepatocytes also commit to the formation of a single apical domain whilst forming the bile canalicular network is not known. The specialized mode of cell division (cf. Chapter 2) suggest that this may not be the case, as it does not appear to be compatible with maintenance of a single apical domain. Also, new lumens could form de novo at the site of cytokinesis. In Chapter 4 we show for the first time that hepatocytes and HepG2 cells have the remarkable ability to generate more than one apical domain per cell. Specifically, ex vivo cultured adult rat primary hepatocytes transiently created more than one apical domain per cell (i.e., a multipolar phenotype) during formation of a bile canalicular-like network. Using HepG2 cells as a model for the developing liver, we further characterized the multipolar phenotype and found that cells developed multipolar phenotypes via a number of mechanisms, though most second lumens were generated de novo or at the site of cytokinesis. Notably, in contrast to primary hepatocytes, HepG2 cells did not proceed to merge lumens and form canalicular structures, and consequently lost one of the lumens. Mostly, cells lost their multipolar phenotype because one of the lumens dissolved or disappeared, suggesting that maintaining multiple apical domains is not a stable state in HepG2 cells. Indeed, HepG2 cells were better able to maintain a monopolar phenotype compared to a multipolar phenotype. In line with this are the observations that apical trafficking components were unequally distributed between lumina, and formation of a second lumen occurred less efficient compared to lumen formation from a non-polarized state. Surprisingly, introduction of Slp2a, which controls the formation of a single apical plasma membrane domain in simple epithelial cells and is not expressed in the liver, did not inhibit HepG2 cells to form a second apical domain per cell. It did however reduce the life time of the multipolar phenotype, albeit via unknown mechanisms. Here, Slp2a likely acts as a dominant negative factor by binding to components of the intracellular trafficking machinery such as syntaxins and muncs that are required for maintenance of apical domains. The finding that Slp2a did not impair HepG2 cells to form a second apical plasma membrane domain can be explained by the localization of Slp2a and its pathway components Rab27, PIP2, PTEN and Cdc42. Whereas these proteins are exclusively apically localized in other epithelia, they were distinctly localized in HepG2 cells. For example, next to localizing apically, Slp2a was also present at the basolateral domain. PIP2, which recruits Slp2a to the plasma membrane in other epithelia, was localized similarly. The distinct mode of apical protein trafficking (that is, first to basolateral domain before reaching the apical domain) in hepatocytes may account for the basolateral components of Slp2a and PIP2 in HepG2 cells, though how PIP2 is

generated at the plasma membrane is not clear, because Cdc42 and PTEN (which recruit PTEN and generate PIP2 at the membrane, respectively, and are apically localized in simple epithelial cells) did not appear to be localized to plasma membrane domains at all. Apart from Slp2a's distinct localization in HepG2 cells, Slp2a did not significantly recruit Rab27 vesicles to the plasma membrane area and most of the Rab27 was in the cytosol. In fact, Rab27(a) did not appear to function in hepatocyte polarity at all, since constitutively active and dominant negative Rab27a expressing HepG2 cells were still able to polarize and formed lateral lumens. Thus, hepatocytes are not limited (that is, committed) to form a single apical domain during lumenogenesis and display a specialized molecular context that is incompatible with simple epithelial trafficking machinery. The formation of multiple apical domains per cell may help form the bile canalicular network, as compared to the elongation of a single apical lumen, the merging of multiple apical lumens is likely to be more effective (and less energy and time consuming) in forming canaliculi that circumvent entire cells. Also, multiple apical poles may provide a spatial cue for lumens to direct the elongation and placement of canaliculi. Apart from hepatocytes, cholangiocytes are likely to utilize a Slp2a-like pathway to generate a single apical domain and lumen during their lumenogenesis, although this has yet to be investigated. Also, the role of Slp5, a liver-specific Slp [211], remains to be explored.

Taken together, the investigations performed in this thesis point out that hepatocytes use lumenogenic mechanisms in a unique and distinct manner to form and maintain their polarized architecture. Specifically, a hepatocyte-specific mode of cell division (i.e., asymmetrical segregation of apical domains), that is (partly) driven by a hepatocyte-specific microenvironment, allows hepatocytes to develop and maintain their polarized architecture. Also, rather than committing to a single central apical domain and lumen, hepatocytes form multiple apical domains and lumina during bile canalicular network formation. These hepatocyte-specific lumenogenic processes are aided by the a distinct mode of intracellular trafficking and unique cellular context (e.g., distinct expression/activity of single lumen-promoting proteins). Consequently, this thesis provides novel insight and ideas into how different epithelial cell polarity types and their corresponding tubular networks are developed in the liver.

PERSPECTIVES

Biological perspectives

In this thesis, we have described intrinsic and extrinsic mechanisms used by hepatocytes to generate and maintain hepatocyte polarity and have proposed their importance for formation of the bile canalicular network. Logically, the next step would be to determine whether the proposed mechanisms indeed serve the formation of the canalicular network in vivo. Studies focused on bile canalicular network formation are however hampered by the fact that, apart from primary hepatocytes, no perfect in vitro model systems are currently available in which hepatocytes form an extensive branched canalicular network during liver development. While primary hepatocytes cultured in collagen sandwich configurations are able to retain most liver functions and form bile canaliculi, they are relatively difficult to manipulate and costly to obtain. Also, these cells are typically not proliferative, and therefore not so suitable for studies on the orientation of cell division and the microenvironment, although it might be possible to stimulate primary hepatocytes to enter the cell cycle and proliferate [93]. Apart from primary hepatocytes, the best cell model for studying the fusion of apical lumens and formation of a canalicular structure is believed to be Can10 cell line, a derivate of rat Fao hepatic cells [97]. Can10 cells are the only hepatic cells that forms apical lumens that can span multiple cells, although not in a chicken wire fashion and it does not circumvent entire cells. The lumen is dilated compared to primary cells and hepatocytes in vivo and it is possible that Can10 may show a form (or mixture) of simple polarity instead of hepatocyte polarity, and would therefore be less suitable for studying bile canalicular network formation.

Ideally, a cell model is needed that is able to go through all developmental stages, i.e. from foregut-specified to fully matured liver cells. HepaRG cells are perhaps suitable for this purpose, but as described earlier (cf. Chapter 1), they are cumbersome to culture and their ability to polarize has not been well described. Recently, major advances have been made in the development of hepatocytes from pluripotent stem cells (PSC) [212–217], and PSC are a promising alternative to hepatic cell lines and may be able to circumvent most of the problems described above. Moreover, PSC may be a good alternative to difficult and costly in vivo studies on cell polarity in the developing liver. One could, for example, differentiate PSC to specific developmental stages that resemble fetal polarity phenotypes in the developing liver (i.e., with apical pockets instead of canaliculi) while maintaining proliferative activity. In this way, development and mainte-

nance of the polarized architecture of hepatocytes can be experimentally combined with cell proliferatory events to study, for example, the role of cell division orientation in bile canalicular network formation. Also, the role of the microenvironment on liver polarity types could be investigated during the various developmental stages.

Even though PSC may be suitable for studying the effect of the mechanisms described in this thesis on bile canalicular network formation, ultimately these mechanisms would need to be assayed in vivo (e.g., in the regenerating liver). It is now relatively simple to generate transgenic mice and to effectively modify liver genetics by using tail vein-injected adenovirus-mediated gene therapy [218,219], or even naked DNA [220–222]. Using (combinations of) these methodologies, the role of the molecular regulators described in this thesis (Par1b, LGN, Slp2a, and their pathway components) and their respective mechanisms (modeling of the microenvironment, mitotic spindle orientation, and generation of multipolar phenotypes) should be studied in vivo with specific attention to the development and maintenance of the polarized architecture of hepatocytes. Par1a [223], Par1b [224], and Par1d [225] knockout mice already exist, though the effect of Par1b on (specifically) hepatocyte polarization has not yet been studied. Importantly, in mammals Par1 has at least four isoforms, and there may be a redundant effect of Par1 isoforms which advocates the need to perform combinatorial and liver-specific knockout studies. Liver knockouts (or knock-ins) of proteins that regulate mitotic spindle orientation, or the generation of a single/multiple apical plasma membrane domain per cell, have not been described, and it would be interesting to study these proteins in relation to the development and maintenance of hepatocyte polarity in vivo.

Medical perspectives

Many proteins that regulate hepatocyte polarity are implicated in the pathogenesis of various liver diseases [19], indicating that epithelial cell polarity and liver disease are intimately linked. The mechanisms described in this thesis are therefore likely to be important for (certain aspects of) liver disease. Defects in mitotic spindle orientation are linked to carcinogenesis in other tissues, and it is possible that mitotic spindle misorientation of proliferating hepatocytes in the liver could contribute to the formation of hepatocellular carcinoma (HCC). Indeed, cystic structures that presumably have arisen from a loss of apicolateral-oriented mitotic spindle orientation and consequently, loss of asymmetric segregation of apical plasma membrane domains, have been reported for HCC [226,227]. Cystic structures are also a common phenotype in polycystic liver

disease, although these fluid-filled structures are usually very large. It is tempting to speculate that mitotically stimulated hepatocytes with altered cell division orientation mechanics could be in some way serve as an origin of these cysts. Also, ductal structures as a response to liver injury and fibrosis (see ref. [124]) may be generated by hepatocytes or hepatocyte-like cells that have lost hepatocyte-specific cell division orientation in response to changes in the cellular microenvironment. These processes may coincide with expression and/or activity of single-lumen stimulating factors such as Slps and their pathway components (a.o. Cdc42, PTEN, and Rab27). These hypotheses should be tested in their respective disease models in rodents and other animal models (e.g., zebrafish [228]).

Understanding how hepatocytes develop and maintain hepatocyte polarity and form the bile canalicular network is also important for the construction of functional liver tissue ex vivo. Importantly, to be able to generate a functional cell system of the liver with a functional bile canalicular network would be very beneficial for testing the toxicology of promising therapeutics, and potentially life-saving for patients with severe liver damage that are waiting for transplantation. Bio-artificial liver (BAL) systems have been attempted before [229,230], though their clinical efficacy needs to be further established. In most cases, the increase in survival of patients with acute liver failure is not substantially increased when subjected to BAL treatment, although progress is being made. Most BALs use primary cells either from patients or animals and it is not clear whether these cells develop a functional bile canalicular network. Notably, as a functional bile canalicular network is important for liver function, BAL systems may gain tremendously from hepatocytic cells that are able to develop a functional biliary system. Patient-derived (induced) PSC grown and expanded ex vivo may be a viable, cost-effective, and relatively safe (i.e., no immunogenicity and unwanted disease transmission) option as a cell source for BALs, and should be included in further research.

The work performed in this thesis provides a significant step into the understanding of the molecular mechanisms and design principles that underlie the development of the polarized architecture of hepatocytes, and, ultimately, the bile canalicular network. Consequently, this work represents a noteworthy contribution to the understanding of the (patho)biology of the liver in health and disease.

APPENDIX

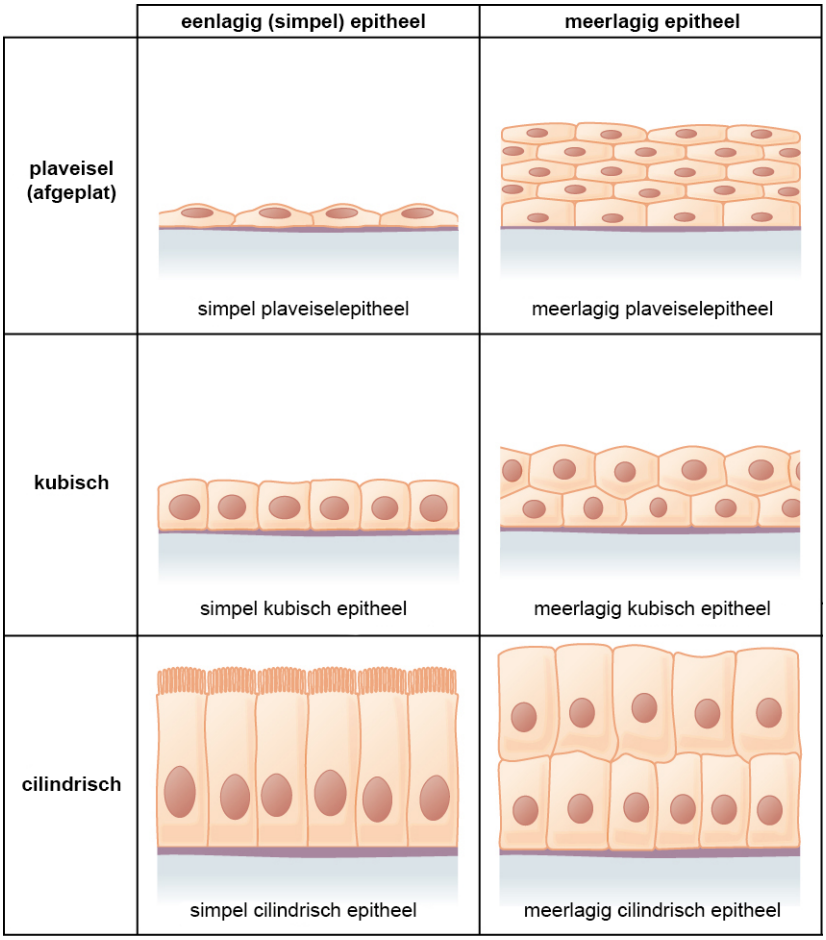
NEDERLANDSE SAMENVATTING

De lever als een buizenorgaan

De lever is het belangrijkste metabole (metabolisme: omzetting of verandering van moleculen) orgaan van het menselijk lichaam. Moleculen die de lever metaboliseert zijn bijvoorbeeld de voedingsstoffen die vrijkomen bij de spijsvertering in de darm. Deze worden opgenomen door de cellen in de darmwand en via het bloed naar de lever getransporteerd. In de lever bevinden zich cellen, hepatocyten (Grieks *hepar-* is lever, *-cyte* is cel), die voedingsstoffen omzetten naar bouwstoffen en energie. De lever heeft ook een sterk ontgiftende werking door schadelijke stoffen (bijvoorbeeld alcohol of pijnstillers) die ons lichaam binnenkomen onschadelijk te maken. Afvalstoffen worden vervolgens via het bloed en de urine afgevoerd, of als ze te schadelijk zijn om via het bloed te worden getransporteerd, via de ontlasting verwijderd. De lever draagt ook bij aan de spijsvertering zelf. Hepatocyten produceren namelijk gal dat galzouten bevat die helpen bij de emulsificatie (mengen van vloeistoffen) van vetten waardoor ze beter worden opgenomen in de darmen. Om gal af te voeren maken de hepatocyten een netwerk van kleine kanaaltjes (als een soort riolering) waarin ze gal kunnen secreteren (uitscheiden) en afvoeren. Dit kanaalnetwerk loopt vervolgens over in grotere galgangen of buizen die uiteindelijk in de galblaas terechtkomen. Als het nodig is, bijvoorbeeld na een maaltijd, wordt gal via de galblaas uitgescheiden in de dunne darm. Gal bevat naast galzouten ook de eerder genoemde afvalproducten die te schadelijk zijn om via het bloed te worden afgevoerd. Gal is in hoge concentraties toxisch voor het lichaam en cellen en het is daarom belangrijk om gal van het bloed te scheiden. Dit is een van de belangrijkste functies van hepatocyten en andere epitheelcellen.

Epitheelcellen zijn belangrijk voor het scheiden van compartimenten

Voordat de organisatie en buisstructuur van hepatocyten wordt besproken is het zaak om eerst een helder beeld van epitheelcellen in het algemeen te schetsen. Epitheelcellen zijn gespecialiseerd in het scheiden van het interne (e.g., bloed of andere cellen) en het externe milieu (bijvoorbeeld lucht in de longen, of de binnenkant van de darmen) van het lichaam. Hiermee beschermen ze het interne milieu tegen schadelijke stoffen en organismen (e.g., bacteriën). Ze hebben naast deze beschermende rol ook een regulerende rol doordat ze selectief moleculen transporteren tussen de twee milieus. Een voorbeeld hiervan is het selectieve proces van vet en suiker opname in de darmen, welke vaak enkel gebeurt in één richting (namelijk van binnenkant darm naar het bloed). Een ander voorbeeld is het selectief opnemen van galzouten uit het bloed (die



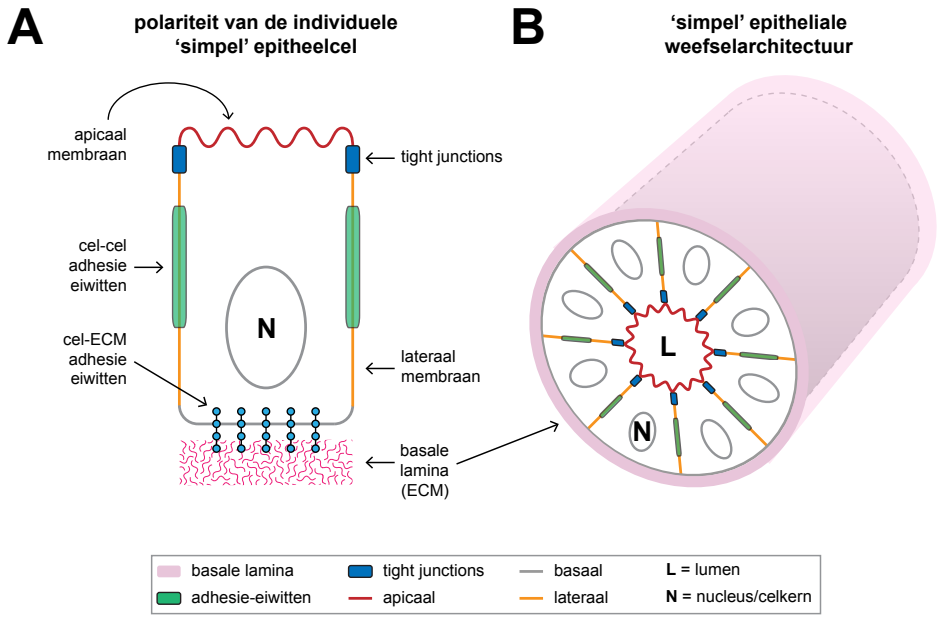
Figuur 1. Epitheelcelllen en hun verschillende varianten. Simpel (i.e., eenlagig) epitheelcellen vormen een monolaag van een enkele cel dik, terwijl meerlagig epitheel bestaat uit meerdere cellagen. Figuur hergebruikt met toestemming van OpenStax College (2013) [130].

samen met vetten uit de darm zijn opgenomen) door hepatocyten in de lever. Naast hepatocyten zijn er verschillende soorten epitheelcellen bekend. Het type dat het meest wordt aangetroffen in het menselijk lichaam wordt ook wel de 'simpele' epitheel cel genoemd (zie Figuur 1). Deze epitheelcellen kenmerken zich vooral doordat ze zijn georganiseerd in cellagen van een enkele cel dik (ook wel monolagen genoemd), die vervolgens om een holte 'gevouwd' zijn en zo effectief de holte (ook wel 'lumen' genoemd; de binnenkant van een buis) afsluiten van de rest van het weefsel (zie Figuur 2).

Epitheelcellen zijn gepolariseerde cellen

Het maken van (simpel) epitheliale monolagen, en het scheiden van twee milieus vereist een (cel)biologisch verschijnsel dat 'polariteit' heet. Dit verschijnsel heeft niks met lading te maken, maar met de niet-gelijke verdeling van cel membranen (ook wel plasma membranen genoemd). Cellen bestaan uit een celkern met erfelijk materiaal (DNA, genen) en organellen zoals bijvoorbeeld mitochondriën (voor de energiehuishouding), die bijeen gehouden worden door celmembranen. Celmembranen bevatten voornamelijk lipiden (i.e., vetten), maar ook andere moleculen zoals eiwitten. Een eiwit, bestaande uit ketens van aminozuren, wordt gemaakt door een gen af te lezen en te vertalen naar een specifieke volgorde van aminozuren die bepaalt welk eiwit er gemaakt wordt. Eiwitten zijn dus de biologisch-actieve producten van onze genen. Eiwitten verzorgen nagenoeg alle cellulaire functies, zoals bijvoorbeeld enzymatische reacties of transport van ionen en andere moleculen, en zijn absoluut onmisbaar. Eiwitten die in plasma membranen zitten zijn bijvoorbeeld transporteiwitten die het transport van moleculen over het celmembraan bewerkstelligen. Zo kan een cel bijvoorbeeld selectief suikermoleculen opnemen om die vervolgens om te zetten naar energie.

In niet-gepolariseerde epitheelcellen zijn eiwitten en lipiden homogeen verdeeld over het celmembraan. Gepolariseerde epitheelcellen maken gespecialiseerde 'domeinen' op hun celmembraan, zoals een apicaal (Latijn *apex*; piek, top) domein wat in contact staat met het lumen (binnenkant van de buis; holte), een basaal (Latijn *basis*; ondersteuning, fundering) domein wat in contact staat met bindweefsel en bloed, en een lateraal (Latijn *lateralis*; betrekking hebbend op de zijkant) domein waarmee ze aan naburige cellen vastzitten (zie Figuur 2A). Elk van deze domeinen heeft een eigen vet- en eiwitsamenstelling die bijdraagt aan de specifieke functie van dat domein. Bijvoorbeeld, het laterale domein wat in contact staat met het laterale domein van een andere cel bevat voornamelijk adhesie-eiwitten die zorgen voor een stevige verbinding en houvast tussen de cellen. Epitheelcellen zorgen er actief voor dat de verschillende celmembraan domeinen niet mengen. Dit doen ze onder andere door 'tight junctions' (een specifiek type eiwitcomplex) te plaatsen op de plekken waar het laterale membraan in het apicale membraan overgaat. Deze tight juntions zorgen tegelijk voor een sterke verbinding tussen de cellen waardoor de epitheliale cellaag ondoordringbaar is voor moleculen en niet lekt. Omdat in simpel epitheel het apicale membraan vaak loodrecht tegenover het basale membraan is geplaatst, kan men spreken van een apicaal-basale 'as'. Daarom wordt het polariteitsverschijnsel in simpel epitheelcellen ook wel apicaal-basale polariteit genoemd. Het is dankzij deze polariteits-as dat simpel epitheelcellen zich kunnen ontwikkelen in monolagen en, uiteindelijk, holle buizen (zie Figuur 2B).



Figuur 2. Cel- en weefselarchitectuur van simpel epitheel. **(A)** Simpel epitheelcellen zijn gepolariseerd. Hun apicale, laterale, en basale membraandomeinen hebben elk een specifieke combinatie van eiwitten en vetten. Apicaal en basaal-laterale membraandomeinen zijn van elkaar geschieden door tight junctions. Basale plasma membraandomeinen staan in contact met de basale lamina die zorgt voor houvast. Adherens junctions en desmosomen zorgen voor cel-cel adhesie (houvast) tussen naburige epitheelcellen. **(B)** Simpel epitheelcellen vormen monolagen die een centraal lumen omsluiten; hiermee vormen ze buizen.

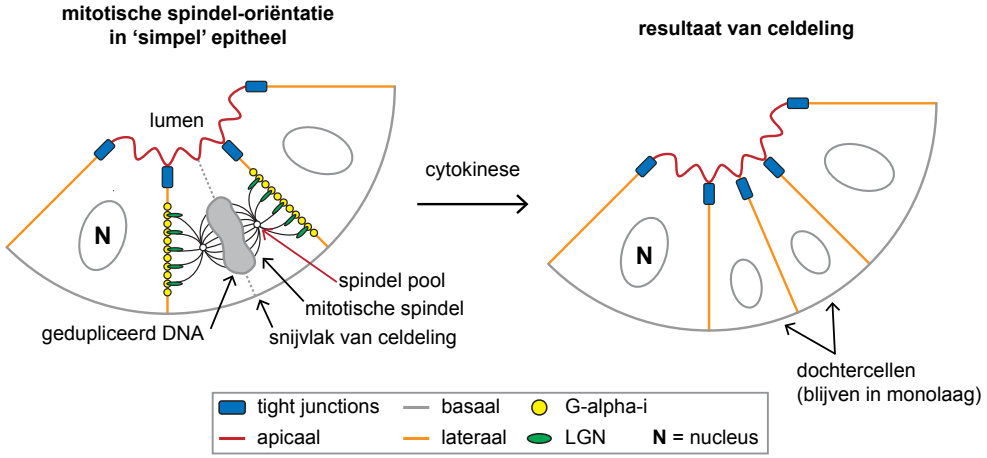
De apicaal-basale polariteits-as coördineert de vorming van buizen

Het hebben van een apicaal-basale polariteits-as stelt epitheelcellen in staat om een buis te vormen. Aan het vormen van een apicaal-basale polariteits-as, en daarna de buis, liggen verschillende processen en mechanismen ten grondslag. Tijdens de ontwikkeling van de apicaal-basaal polariteits-as (en de buis) wordt er door een tweetal niet-gepolariseerde epitheelcellen een apicaal plasma membraan gevormd op de plek waar ze aan elkaar vast zitten. Doordat er op het apicale membraan transport eiwitten zitten die ionen kunnen transporteren, kan er (door osmose) vloeistof in de ruimte tussen de twee apicale domeinen worden gedeponeerd. Hierdoor ontstaat er een vloeistof gevulde holte tussen twee cellen. Een belangrijk mechanisme voor het maken van apicale domeinen en holtes is gericht intracellulair eiwit transport. Eiwitten en vetten worden in cellen niet willekeurig door de cel getransporteerd, maar worden via sorteersystemen 'gesorteerd' en getransporteerd naar specifieke plekken in de cel zoals het apicale celmembraan domein. Hierdoor blijft de identiteit (i.e., de eiwit en lipid samenstelling) en functie van deze membraan domeinen intact. Ook zorgen cellen

er op deze manier voor dat er niet op andere plekken apicale eiwitten en lipiden terecht komen (die daar vervolgens een apicaal membraandomein en lumen kunnen vormen, wat niet de bedoeling is). Gericht eiwit transport is dus erg belangrijk voor het vormen en in stand houden van celmembraan domeinen, en daarme het genereren van apicaal-basale polariteit.

Een belangrijk mechanisme wat er voor zorgt dat een kleine holte (lumen) omsloten door twee cellen uit kan groeien naar een holle buis is celdeling. Wanneer een lumen is gevormd, delen de epitheelcellen op zo'n manier dat de nieuwe (dochter) cellen altijd in contact blijven met het apicale domein, en daarmee dus het lumen (zie Figuur 3). Dit fenomeen wordt ook wel symmetrisch geörienteerde celdeling en/of symmetrische segregatie van celmembraan domeinen genoemd. Niet alleen tijdens de vorming van buizen, maar ook voor de in stand houding van buizen is dit een logisch mechanisme, want zo blijft de integriteit van de buis intact en delen cellen niet 'in' of 'uit' de monolaag van cellen (dit gaat overigens fout in ziektes zoals kanker, waar cellen uit hun context (in dit geval de monolaag) kunnen delen en zo gaan 'zwerven' in het lichaam). De oriëntatie van celdeling wordt voornamelijk bepaald door een selectieve membraan-locatie van eiwitten (zoals $G\alpha_i$ en LGN, zie Hoofdstuk 1) die de mitotische (Grieks *mitose*; draad) spindel (de 'draad' structuur die vermenigvuldigd DNA uit elkaar trekt en evenredig verdeelt over de dochtercellen) kunnen oriënteren door spindel polen (waaruit de draadstructuren komen) te 'vangen' (zie Figuur 3, linker kolom). De oriëntatie van de mitotische spindel bepaalt het 'snijvlak' van celdeling en hoe de membraan domeinen worden verdeeld over de twee nieuwe dochtercellen. In buizen gemaakt door simpel epitheelcellen zijn spindel-oriënterende eiwitten selectief gelokaliseerd op de laterale domeinen, wat ervoor zorgt dat de mitotische spindel loodrecht op de apicaal-basale polariteits-as komt te staan. Dit resulteert vervolgens in de evenredige (symmetrische) verdeling van het apicale membraandomein naar beide dochtercellen (zie Figuur 3, rechter kolom). Kortom, georiënteerde celdeling is belangrijk voor de vorming en in stand houding van epitheliale monolagen en buizen.

Een derde belangrijk mechanisme of factor in het vormen van simpel epitheliale buizen is de extracellulaire matrix (ECM). Simpel epitheelcellen zitten vast (via hun basale membranen) aan een specifiek type ECM: de basale lamina (Engels *basement membrane*, zie Figuur 2). Deze basale lamina zorgt voor mechanische ondersteuning van cellen, maar geeft epitheelcellen tegelijk een signaal om hun apicaal-basaal polariteits-as correct te positioneren. Simpel epitheelcellen positioneren hun apicale plasma membraan vrijwel altijd recht tegenover de plek van de basale lamina. Hierdoor



Figuur 3. Simpel epitheelcellen delen symmetrisch om hun typische weefselstructuur (i.e., buizen) te genereren en te waarborgen. Tijdens mitosis bevindt het eiwit $G\alpha_i$ zich op de laterale membraandomeinen van de delende cel; hier recruteert $G\alpha_i$ het eiwit LGN. LGN bindt vervolgens aan het eiwit NuMA, dat op de astrale microtubules van de mitotische spindel zit. Door aan deze tubules te trekken kan de cel zijn spindel oriënteren. Tijdens cytokinese (de afsplitsing van twee dochtercellen) is, door de oriëntatie van de spindel, het snijvlak van celdeling zodanig geörienteerd dat de cel zijn membraandomeinen symmetrisch (evenredig) verdeelt over zijn twee dochtercellen.

is de basale lamina ook (uiteindelijk) verantwoordelijk voor het oriënteren van de miotische spindel en celdeling, want die wordt immers deels bepaald door de apicaal-basale polariteits-as. Dus, de micro-omgeving van cellen (i.e., de ECM) is een belangrijke extrinsieke factor in het genereren van simpel epitheliale polariteit en de vorming van simpel epitheliale buizen.

Alle bovenstaande processen (intracellulair transport, celdeling, en de micro-omgeving) hebben een gemeenschappelijk doel: een enkele holte (lumen) uit laten groeien tot een grotere holte die omringd is door een enkele (mono)laag van epitheelcellen.

De lever heeft twee verschillende buis-systemen

Zoals eerder besproken (zie eerste paragraaf) maakt de lever een buizensysteem om gal af te voeren. Alvorens dit buizensysteem in meer detail te bespreken, is het noodzaak om kort de weefselarchitectuur van de lever te introduceren. De functionele 'unit' van de lever is de leverlobule (zie Figuur 4A). De lobule heeft ruwweg een hexagonale vorm met in het centrum de centrale ader. Op elke hoek van de lobule bevinden zich een drietal vaten die de portale 'triade' wordt genoemd: een portale ader, een slagader, en een galbuis. De meest voorkomende cel in de lobule is de hepatocyt die in platen van een enkele (of soms dubbele) laag cellen is gesitueerd. Bloed komt de lobule binnen

via de portale ader en loopt via een netwerk van sinusoïdale capillairtjes naar de centrale ader. De platen van hepatocyten zijn zodanig geplaatst dat er aan weerszijden van de plaat een sinusoïd bevindt. Deze weefselarchitectuur maakt de uitwisseling van moleculen tussen het bloed en de hepatocyten erg efficiënt.

De galbuizen van de portale triade wordt gevormd door simpel epitheelcellen die cholangiocyten worden genoemd. Deze epitheelcellen vormen net als simpel epitheelcellen een enkele cellaag om een holte, zijn omringd door een basale lamina, en vertonen een simpel epitheliale apicaal-basale polariteits-as. Echter, hepatocyten (die het gal produceren, zie eerdere paragraaf) maken een buizensysteem bestaande uit kleine kanaaltjes (canaliculi genoemd) dat anatomisch gezien fundamenteel verschillend is van het galbuissysteem van cholangiocyten (Engels bile canaliculi en bile ducts; zie Figuur 4B). Dit canaliculaire netwerk kenmerkt zich vooral doordat het ontzettend vertakt is en vaak complete hepatocyten omsluit als in een soort 'kippengaas'-structuur. Ook wordt, op celniveau, het kanaaltje op elk moment maar door twee hepatocyten gedeeld, in plaats van door een monolaag van meerdere cellen zoals in galbuizen. De anatomie van het gal canaliculaire netwerk vindt zijn oorsprong in een specifiek type celpolariteit van hepatocyten. Hepatocyten bevinden zich in platen en staan in contact met het bloed in de sinusoïden met hun (meerdere) basale membranen. Met hun laterale domeinen zitten ze, net als andere epitheelcellen, aan elkaar vast. Op deze plek plaatsen ze tevens hun apicale membraan en lumen, omsloten door tight junctions. Deze specifieke architectuur van de hepatocyt staat in de literatuur bekend als hepatocyt-polariteit (zie Figuur 5A). Het (kleine) lumen is vervolgens in de volwassen lever uitgegroeid naar een klein galkanaaltje wat tussen de hepatocyten door loopt (zie Figuren 4B en 5B). De galkanaaltjes komen uiteindelijk uit in de groteren galgangen. De lever waarborgt dus twee type epitheelcellen (hepatocyten en cholangiocyten) die beide een verschillende gepolariseerde architectuur hebben, en een specifiek type buis (of in het geval van de hepatocyt, kanaaltje) vormen (vergelijk Figuur 5A,B met 5C,D).

De regulatie van hepatocyt polariteit en het gal canaliculaire netwerk

De vorming van een gal canaliculair netwerk is belangrijk voor de lever om zijn functie goed uit te kunnen voeren. Obstructie of malformatie van galkanaaltjes heeft in het ergste geval leverfalen tot gevolg. Het is daarom belangrijk te weten hoe hepatocyt polariteit en het gal canaliculaire netwerk tot stand komt en hoe het wordt onderhouden. Zoals eerder beschreven, dragen factoren zoals de oriëntatie van celdeling, de micro-omgeving, en intracellulair transport bij aan de vorming van buizen. Deze mechanismen

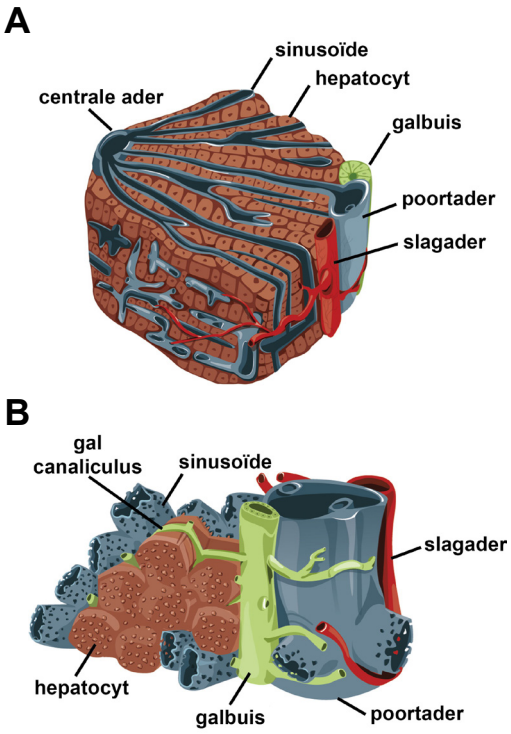
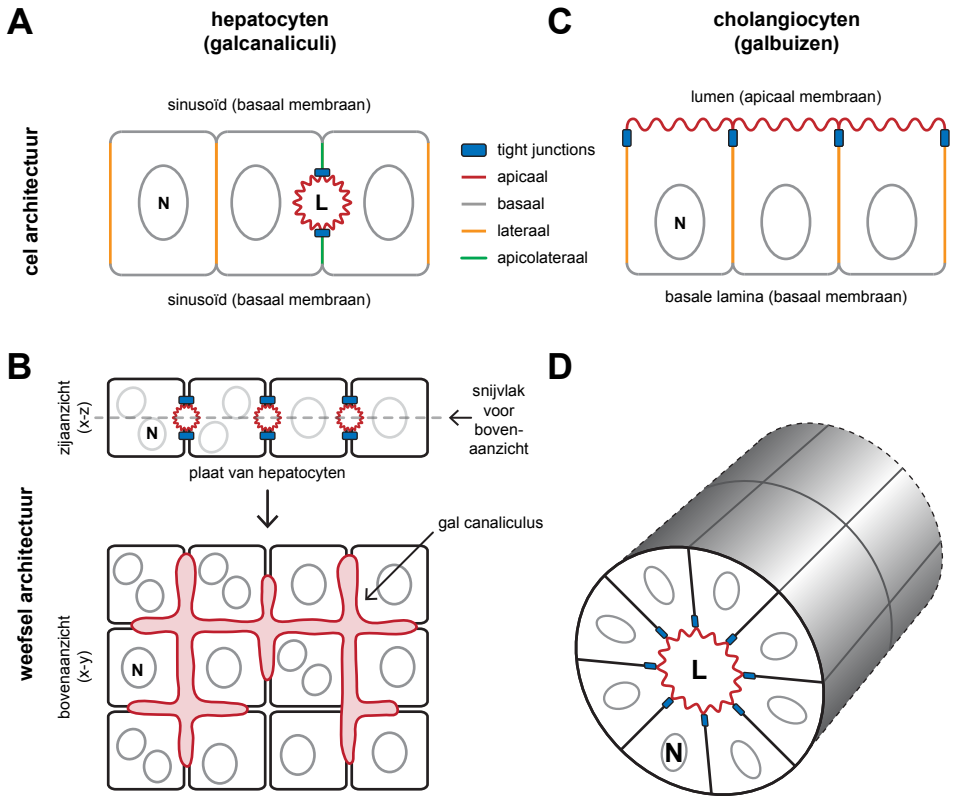


Figure 4. Weefselarchitectuur van de lever. **(A)** Anatomoie van de leverlobule. Bloed stroomt van de poortader en slagader door kleine cappilairen (sinusoïden) weg naar de centrale ader. Hepatocyten zitten en platen en wisselen moleculen uit met het bloed. **(B)** Hepatocyten vormen een galcanaliculair netwerk waarin ze gal uitscheiden. Gal stroomt vervolgens naar grotere galbuizen die uiteindelijk uitkomen in de galblaas en darm. Figuur hergebruikt met toestemming van Elsevier [87], © 2010.

worden geleid door de (simpel epitheliale) apicaal-basale polariteits-as, die als een intern 'kompas' fungeert. Daarnaast zijn al deze mechanismen toegespitst op het genereren en behouden van een enkel centraal lumen. Hepatocyten hebben echter een ander type celpolariteit, en hoe processen zoals celdeling en intracellulair transport in hepatocyten geregeld zijn en bijdragen aan hepatocyt-polariteit (en daarmee de vorming van galkanaaltjes) is niet bekend. Daarom hebben we in dit proefschrift geprobeerd de volgende vragen te beantwoorden: a) hoe wordt de oriëntatie van celdeling gebruikt door hepatocyten om celpolariteit en galkanaaltjes te vormen, b) welke rol speelt de micro-omgeving in hepatocyt-polariteit, en c) zijn hepatocyten gelimiteerd tot het maken van een enkel apicaal domein en lumen? Ook hebben we door deze vragen te beantwoorden getracht inzicht te krijgen in hoe verschillende types van celpolariteit en buissystemen in de lever ontstaan.



Figuur 5. Hepatocyten vormen een uniek type celpolariteit en buis/kanaalarchitectuur. **(A)** Hepatocyten vormen een apicaal membraandomein en lumen tussen (maar) twee naburige hepatocyten. Aan weerszijden staan hepatocyten in contact met de sinusoïden en bloed (basaal membraandomeinen). **(B)** Hepatocyten vormen een uniek buizensysteem: het galcanaliculaire netwerk. Dit systeem vormt een uitgebreid netwerk dat door de levermassa is verweven en individuele cellen volledig kan omsluiten. **(C)** Gepolariseerde architectuur van simpel epitheelcellen. **(D)** Weefsel- en buisarchitectuur gemaakt door simpel epitheelcellen. L = lumen. N = nucleus.

a) Hepatocyten segregeren apicale domeinen asymmetrisch tijdens celdeling

In simpel epitheelcellen is de manier waarop cellen delen tijdens de ontwikkeling streng gereguleerd, want het is belangrijk dat de verschillende membraan domeinen (apicaal, lateraal, en basaal) intact blijven bij celdeling. Het canaliculaire netwerk van de lever is echter zodanig gecompliceerd dat celdeling anders gereguleerd moet zijn dan in simpel epitheliale weefsels (zoals in de darm en long). Een symmetrische celdeling en segregatie van apicale plasma membraan domeinen zou in hepatocyten leiden tot de vorming van uitsluitend grotere buizen zoals galgangen. Ook zou hepatocyt-polariteit hierdoor verloren gaan. In Hoofdstuk 2 laten we zien dat hepatocyten een andere manier van georiënteerde

celdeling hebben en dat dit gereguleerd wordt door het eiwit Par1b, die met name de lokalisatie van eiwitten die betrokken zijn bij het oriënteren van de celdeling reguleert. Deze eiwitten (e.g., LGN) zijn in regenererend lever weefsel en HepG2 cellen (een modelsysteem voor ontwikkelende levercellen) gelokaliseerd op een hepatocyt-specifiek membraan domein: het apicolaterale membraan. Dit domein bevindt zich vlak naast het apicale lumen en de tight junctions. Door eiwitten als LGN hier te plaatsen, zorgen hepatocyten ervoor dat een van de spindel polen hier naar toe worden georiënteerd, wat uiteindelijk resulteert in een asymmetrische segregatie van apicale plasma membraan domeinen (in tegenstelling tot simpel epitheel). Het uitschakelen van Par1b in HepG2 cellen induceert inderdaad de vorming van galgang-achtige structuren, hoofdzakelijk geïnduceerd door verlies van de apicolaterale spindel pool oriëntatie en inductie van symmetrische segregatie van apicale domeinen. Hiermee is Par1b dus een belangrijke factor in het reguleren van celpolariteit van hepatocyten, maar daarmee ook van verschillende types epitheel in de lever.

b) Blootstelling van hepatocyten aan een basale lamina induceert cholangiocyt-celpolariteit en weefselanatomie

Simpel epitheelcellen zoals cholangiocyten bevinden zich normaliter op een basale lamina (een type ECM) die voornamelijk voor adhesie (houvast) en rigiditeit zorgt. De basale lamina reguleert tevens de (apicaal-basaal) polarisatie en oriëntatie van celdeling en is daarmee belangrijk voor de vorming van buizen zoals galgangen. Hepatocyten staan ook in contact met een ECM (in de sinusoïd), maar deze ECM heeft een andere samenstelling dan de basale lamina (basale lamina component laminine is bijvoorbeeld afwezig). In Hoofdstuk 3 laten we zien dat blootstelling van hepatocyten aan een basale lamina-achtige ECM een conversie van hepatocyt-naar-cholangiocyt type celpolariteit induceert. Vooral het eiwit laminine is hiervoor belangrijk. De oriëntatie van celdeling is mede verantwoordelijk voor de conversie en wederom afhankelijk van het mitotische spindel-oriënterende eiwit LGN. Blootstelling van HepG2 cellen aan een basale lamina zorgt voor een re-lokalisatie van Gαi en LGN weg van het apicolaterale membraan, wat symmetrische celdeling induceert. LGN is belangrijk in de fenotypische conversie, want uitschakeling van LGN vermindert het aantal gevormde cholangiocyt-achtige celstructuren wanneer hepatocyten gegroeid worden op een basale lamina. De selectieve micro-omgeving van hepatocyten is dus belangrijk voor het vormen van hepatocyt polariteit en galkanaaltjes,

en tevens een belangrijke factor in het genereren van verschillende types celpolariteit en buissystemen in de lever. We speculeren dat Par1b (zie Hoofdstuk 1) de lokalisatie van mitotische spindeleiwitten reguleert (Hoofdstuk 2) door de depositie, compositie, en signaaltransductie van de ECM te beïnvloeden.

c) Hepatocyten maken meerdere apicale domeinen per cel

Tijdens de ontwikkeling van simpel epitheliale buizen staat het genereren van een enkel lumen centraal. Dit wordt onder andere bewerkstelligd door georiënteerde celdeling (i.e., symmetrische segregatie van plasma membraan domeinen). Naast celdeling zijn ook intracellulair transport eiwitten zoals synaptotagmin-like protein 2a (Slp2a) belangrijk bij het maken en behouden van een enkel apicaal domein door ervoor te zorgen dat er maar op één plek een apicaal domein wordt gevormd. Uitschakeling van Slp2a gaat gepaard met verlies van een enkel centraal lumen en malformatie van de uiteindelijke buis. De reden hiervoor is dat Slp2a deletie ervoor zorgt dat er tijdens de ontwikkeling op meerdere plekken apicale domeinen gegenereerd worden. Hierdoor raken andere lumen-vormende mechanismen zoals celdeling mogelijk in de war, aangezien er niet meer een enkele apicaal-basale polariteits-as bestaat in deze cellen. Voor hepatocyten is het echter onduidelijk of ze een enkel apicaal domein en lumen per cel maken tijdens het vormen van het gal canaliculaire netwerk, of dat ze er meerdere kunnen vormen. De afwezigheid van Slp2a in de lever en de asymmetrische celdeling van hepatocyten wijzen erop dat hepatocyten wellicht niet gelimiteerd zijn tot het maken van een enkel apicaal domein per cel, en meerdere apicale domeinen en lumens per cel kunnen vormen. In Hoofdstuk 4 tonen we aan dat hepatocyten inderdaad meerdere apicale domeinen per cel kunnen maken. In primaire hepatocyten (geïsoleerd uit een volwassen lever) is het vormen van meerdere apicale domeinen een intermediair fenotype tijdens het maken van een gal canaliculair netwerk. HepG2 cellen kunnen ook meerdere apicale domeinen per cel maken, maar kunnen deze niet laten uitgroeien tot een netwerk. HepG2 cellen kunnen een multipolair fenotype niet oneindig lang volhouden en verliezen uiteindelijk een van de domeinen en lumens. Dit komt waarschijnlijk doordat het voor deze cellen moeilijk is om apicaal transport componenten evenwichtig te verdelen over meerdere apicale domeinen. Daarnaast is de vorming van een tweede apicaal domein en lumen niet zo effectief als het vormen van een nieuw lumen vanuit een niet-gepolariseerde toestand. Introductie van Slp2a in HepG2 cellen voorkomt niet de vorming van meerdere apicale domeinen, maar zorgt

er alleen voor dat HepG2 cellen een multipolair fenotype minder lang kunnen volhouden. De reden waarom Slp2a in hepatocyten een andere functie heeft als in simpel epitheelcellen is waarschijnlijk dat hepatocyten een andere manier van apicaal eiwit transport hebben ontwikkeld die het niet toe laat om apicale eiwitten direct naar een apicaal eiwit de transporteren. Hierdoor zijn eiwitten en lipiden (zoals PIP2) die Slp2a rekruteren naar het apicale domein (waar het zijn werk doet), net als Slp2a zelf, niet selectief gelokaliseerd op het apicale domein en naast een apicale lokalisatie ook terug te vinden op de laterale membranen. Dit laat zien dat, naast het feit dat hepatocyten niet gelimiteerd zijn tot het maken van een enkel apicaal domein en lumen per cel, hepatocyten een unieke cellulaire context hebben die waarschijnlijk bijdraagt aan het efficiënt en correct maken van het gal canaliculaire netwerk vanuit meerdere apicale lumens per cel (e.g. fuseren van meerdere domeinen).

Samengevat laat dit proefschrift voor het eerst zien dat de oriëntatie van celdeling anders gereguleerd is in hepatocyten, dat de specifieke samenstelling van de ECM rond hepatocyten grote invloed heeft op celpolariteit (o.a. door het reguleren van georiënteerde celdeling), en dat hepatocyten meerdere apicale domeinen en lumens per cel kunnen vormen. Dit laatste wordt mogelijk wordt gemaakt door een specifieke cellulaire context (e.g., de afwezigheid van eiwitten als Slp2a) in hepatocyten. Het maken van meerdere apicale domeinen wordt tegelijkertijd mogelijk gemaakt door het asymmetrisch segregeren van apicale domeinen waarbij nieuwe lumens kunnen worden gevormd op de plaats van cytokinese (zie Hoofdstuk 2). De unieke manier van celdeling wordt vervolgens mede gereguleerd door onder andere de specifieke compositie van de hepatocyt micro-omgeving. Al deze processen dragen ertoe bij dat hepatocyten een uniek type van celpolariteit en uiteindelijk het (voor de lever) unieke gal canaliculaire netwerk kunnen vormen en onderhouden. Ook dragen deze processen bij aan het genereren van de verschillende types epitheelcellen en buissystemen in de lever.

Perspectieven

De volgende stappen in het hier beschreven onderzoek zullen gericht moeten zijn op het in vivo testen van de in dit proefschrift aangetoonde mechanismen. Dit proefschrift brengt naast inzicht in de ontwikkelingsbiologie van de lever ook belangrijke nieuwe inzichten en pathologische mechanismen voor ziektemodellen zoals bijvoorbeeld polycystic liver disease (cystelever), fibrose, en hepatocellulair carcinoma. In deze

ziektebeelden zijn veranderingen in buis-structuren te zien waarvan het niet helemaal duidelijk is wat de oorzaak en cellulaire oorsprong is (hepatocyt of cholangiocyt?). Met name een veranderende micro-omgeving gecombineerd met stimulatie van proliferatieve activiteit en symmetrische celdeling zou een belangrijke factor kunnen zijn in het ontstaan van celstructuren in deze ziektes. Dit proefschrift heeft ook implicaties voor het creëren van functioneel lever weefsel ex vivo (buiten het lichaam) met een functioneel gal canaliculair netwerk. Dit is met name bevorderlijk voor het maken van bijvoorbeeld bio-artificiële levers om mensen met leverfalen (die bijvoorbeeld wachten op transplantatie) beter te kunnen behandelen dan nu mogelijk is. Ook kan zo'n systeem worden ingezet om nauwkeuriger en betrouwbaarder de toxiciteit van bijvoorbeeld geneesmiddelen te testen. Uiteindelijk zal onderzoek naar de regulatie van celpolariteit en buisvorming in de lever bijdragen aan onze kennis over hoe de unieke leverarchitectuur tot stand komt, en ons nieuwe inzichten geven met betrekking tot leverziektes en mogelijke therapeutische toepassingen.

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"This is the real secret of life – to be completely engaged with what you are doing in the here and now. And instead of calling it work, realize it is play."

— Alan W. Watts

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ABBREVIATIONS

ABCB1 – ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1
ABCC2 – ATP-Binding Cassette, Sub-Family C (CFTR/MRP), Member 2
AMPK – Adenosine Monophosphate-activated Protein Kinase
AP1 – Adaptor Protein 1
aPKC – atypical Protein Kinase C
BAL – Bio-Artifical Liver
BC – Bile Canaliculus
BME – Basement Membrane Extract
BMP – Bone Morphogenetic Protein
BSEP - Bile Salt Export Pump
cAMP – cyclic Adenosine Monophosphate
Dlg - Discs large
DPPIV – Di-Peptidyl Peptidase-4
ECM – Extra-Cellular Matrix
FGF - Fibroblast Growth Factor
$G\alpha_i$ – Gi Alpha Subunit
GEF – Guanine Exchange Factor
GPI - Glycophosphatidylinositol
GTP - Guanosine Tri-Phosphate
H2B – Histone-2B
HNF- Hepatocyte Nuclear Factor
Lgl - Lethal giant larvae
LGN – Leu-Gly-Asn-repeat protein
MDCK – Madin-Darby Canine Kidney
NuMA – Nuclear Mitotic Apparatus protein
PA – Polarity Axis
PALS – Proteins Associated with Lin-7
PAR - Partitioning-defective
PATJ - PALS1-Associated TJ protein
PTEN - Phosphatase and Tensin homolog
PIP – Phosphatidyl-Inositol Phosphate
PKA – Protein Kinase A
PSC – Pluripotent Stem Cell
SA – spindle axis
TPR – Tetratrico-Peptide Repeat
ZO-1 – Zona Occludens-1